THE ROLE OF POLYCOMB REPRESSIVE COMPLEX 2 IN EPIDERMAL HOMEOSTASIS AND HAIR GROWTH

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Abstract

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Keywords: Skin, epidermis, hair follicle, Polycomb group proteins, EZH2, differentiation

Polycomb repressive complex 2 (PRC2) catalyses the methylation of 'Lys-27' of histone H3, leading to transcriptional repression of target genes through its catalytic subunit Enhancer of zeste homolog 1/2 (EZH1/2). PRC2 functions as a critical regulator of stem cells in mouse embryonic and adult tissues. However, the role of PRC2 in human skin remains largely unknown.

This study investigated the role of PRC2 in human epidermal homeostasis and hair growth. The expression of EZH2 was elevated in differentiating suprabasal layers of the human epidermis. Consistently, EZH1/2 expression and enzymatic activity was upregulated in differentiating primary human keratinocytes (NHEKs) *in vitro*. Inhibition of EZH2 and Embryonic ectoderm development (EED) in NHEKs stimulated the expression of differentiation-associated genes, therefore leading to their premature differentiation; while inhibition of EZH1/2 reduced cell proliferation and promoted apoptosis. Silencing of EZH2 in NHEKs induced complex changes in gene expression programmes, including the upregulation of terminal differentiation genes, such as Filaggrin. EZH2 expression was downregulated in aged keratinocytes accompanied with upregulation of senescence-associated

genes, *p16*^{INK4A} and *p19*^{INK4D}, suggesting EZH2 involvement in epidermal aging.

In human anagen hair follicle (HF), EZH2 was detected in stem and progenitor cells; and hair matrix keratinocytes. Silencing EZH2 in HFs accelerated anagen-catagen transition and retarded hair growth accompanied by decreased proliferation and increased apoptosis. Silencing EZH2 in outer root sheath keratinocytes resulted in upregulation of *p14*^{ARF}*and K15*, suggesting EZH2 involvement in regulating proliferation and stem cell activity.

Thus, this study demonstrates that PRC2-mediated repression is crucial for epidermal homeostasis and hair growth. Modulating the activities of PRC2 in skin might offer a new therapeutic approach for disorders of epidermal differentiation and hair growth.

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Abbreviations

AEBP2	Adipocytes Enhancer-binding protein 2
ANCR	Anti-differentiation non-coding RNA
AP-1	Activator protein 1
BMI-1	B lymphoma Mo-MLV insertion region 1 homolog
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium Chloride
CBX	Chromobox homolog
cDNA	Complementary Deoxyribonucleic Acid
CTS	Connective tissue sheath
D	Differentiated
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DPBS	Dulbecco’s phosphate-buffered saline
DP	Dermal papilla

DTT	1, 4-Dithiothreitol
DZNep	3-Deazaneplanocin A
EGCG	Epigallocatechin-3-gallate
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EED	Embryonic Ectoderm Development
EZH1	Enhancer of Zeste Homolog 1
EZH2	Enhancer of Zeste Homolog 2
FBS	Fetal Bovine serum
FDR	False discovery rate
FLG	Filaggrin
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HF/HFs	Hair follicle/ Hair follicles
HFOC	Hair follicle organ culture
HRP	Horseradish peroxidase
H2AK119ub1	Histone H2A mono-ubiquitination on lysine 119

H3K27me3	Histone H3 trimethylation on lysine 27
H3S28p	Phosphorylated Serine 28 of Histone H3
HMT/HMTase	Histone Methyltransferase
IRS	Inner root sheath
JARID2	Jumonji and AT-Rich Interaction Domain Containing 2
JMJD3	Jumonji Domain-containing protein 3
KBM2	Keratinocyte Basal Medium 2
KLFs	Krüppel-like factors
KRT1/K1	Keratin 1
KRT10/K10	Keratin 10
KRT14/K14	Keratin 14
KRT15/K15	Keratin 15
KRT19/K19	Keratin 19
LncRNA	Long non-coding RNA
LOR	Loricrin
mRNA	Messenger RNA
ncRNA	Non-coding RNA
NHEKs	Normal human epidermal keratinocytes
NSG	NOD scid gamma
OD	Optical density

ORS	Outer root sheath
ORSKs	Outer root sheath keratinocytes
PBS	Phosphate buffered saline
PEI	Polyethylenimine
PFA	Paraformaldehyde
PI	Propidium Iodide
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
RNA	Ribonucleic acid
RPM/rpm	Rotations per minute
RT	Room temperature
RT – qPCR	Real time quantitative polymerase chain reaction
SEM	Standard error of mean
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SPRRs	Small Proline Rich Proteins
T°	Temperature

TBST	Tris-buffered saline Tween-20
TDG	Thymine DNA glycosylase
TE	Trypsin/EDTA
TET	Ten eleven translocation
TINCR	Tissue Differentiation-Inducing Non-protein coding RNA
UD	Undifferentiated
UHRF1	Ubiquitin like with PHD and ring finger domains 1
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

CHAPTER 1: INTRODUCTION

1.1 Functions of the skin

Mammalian skin covers a surface area of about $2m^2$ accounting for about 16% of total body weight and is essential for the survival of an organism (Tobin, 2006; Wickett and Visscher, 2006). It acts as coverage and a barrier protecting the internal organs of the body from the external environment. It also acts as a protective barrier against fluid loss, toxic chemical exposure, infectious agents, mechanical damage and ultraviolet radiation (Costin and Hearing, 2007; Tobin, 2006). It plays important roles in thermoregulation by secreting sweat and constricting blood vessels thereby increasing or reducing heat loss (Fuchs, 2007; Koster and Roop, 2007). Sensory receptors in the skin enable an organism to detect touch, temperature, pain, pressure and vibration. Skin can synthesize vitamin D_3 from cholesterol upon direct contact with sunlight. This enables the skin to perform an endocrine function (Lips, 2006). The skin also plays an important role in terms of appearance and social interactions. In addition to all the above functions, the skin is also a self-renewing organ that can heal itself upon injury (Segre, 2006). An array of cell types in the skin enables it to perform these diverse functions. The cell types in the skin include keratin producing keratinocytes, collagen synthesizing fibroblasts, melanin producing melanocytes, energy storing adipocytes, somatosensory Merkel cells and immune cells such as Langerhans cells (Costin and Hearing, 2007; Heath and Carbone, 2013;

Tobin, 2006). In addition, the skin is enriched with various stem cells populations that include $\alpha6^{+}/\beta1^{+}/CD71^{-}$ epidermal stem cells, $CD200^{+}/K15^{+}/K19^{+}$ hair follicle stem cells (HFSCs) in the bulge and c-kit^{+} hair follicle melanocyte stem cells (Botchkareva et al., 2001; Inoue et al., 2009; Li et al., 1998; Nishimura et al., 2002).

Skin appendages include eccrine and apocrine sweat glands, hair, hair follicles, sebaceous glands and nails (Tobin, 2006).

1.2. Structure of the skin

The skin in mammals is a multi-layered organ that consists of three main layers namely:

- The epidermis, largely made up of specialized epithelial cells known as keratinocytes; other cells types include melanocytes, Langerhans cells and Merkel cells;
- The dermis, largely made up of mesenchymal cells such as fibroblasts; other cell types include mast cells, telocytes and macrophages;
- The hypodermis or subcutis, largely made up of adipose tissues (adipocytes) (Fuchs, 2007; McGrath and Uitto, 2016; Tobin, 2006).

The epidermis is stratified into four layers and the dermis consists of two layers (Figure 1.1).

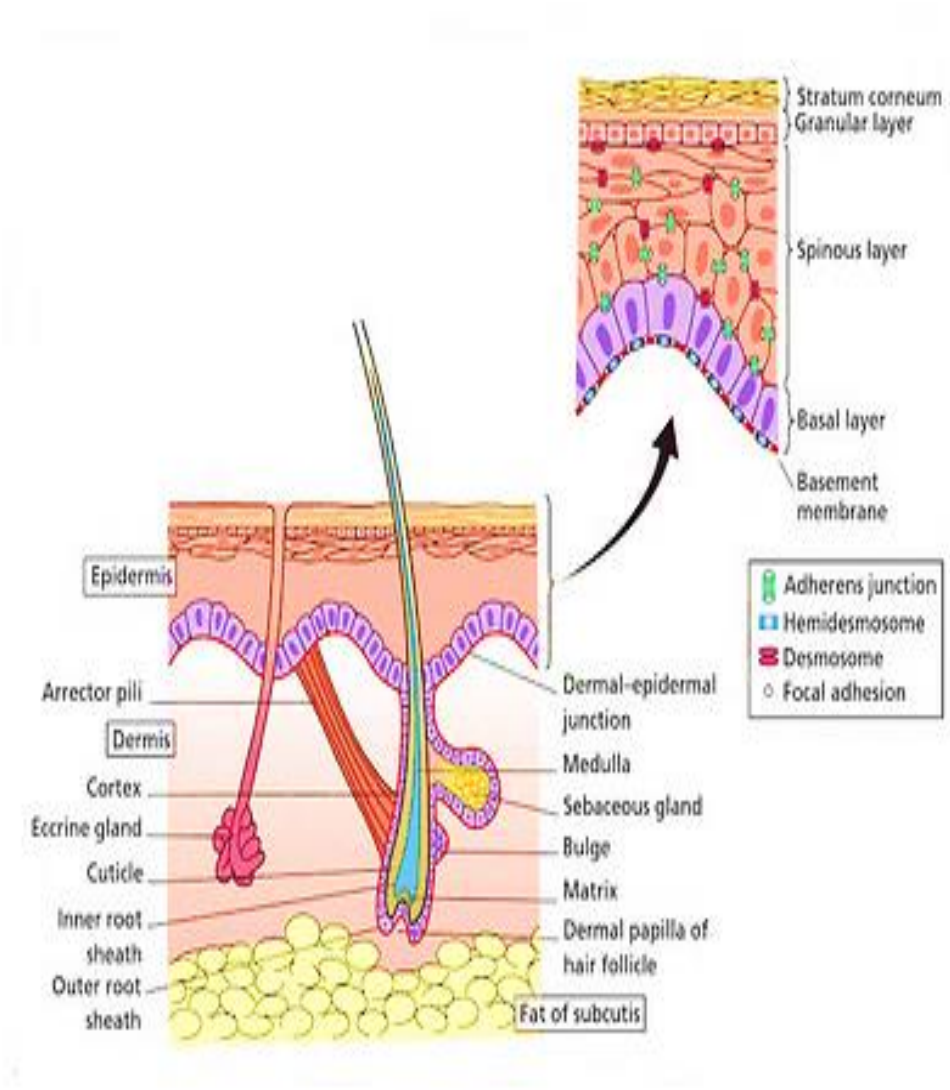


Figure 1.1 – **The human skin.** A diagram of human skin showing the four layers of the epidermis, the dermis, fat of subcutis (Hypodermis) and skin appendages (McGrath and Uitto, 2016)

1.2.1. Epidermis

During embryonic development, a single layer of epidermal progenitor keratinocytes give rise to the epidermis, hair follicle and sebaceous gland through various regulatory pathways such as Notch and Wnt signalling pathways (Yi and Fuchs, 2010). Mammalian epidermis is a stratified squamous epithelium (Figure 1.1) that retains the ability to self-renew under homeostatic and injury conditions by maintaining a population of mitotically active cells in the hair follicle and innermost basal layer (Segre, 2006). Under homeostatic conditions, these mitotically active cells exit the innermost basal layer, stop proliferating and undergo terminal differentiation as they move through the 3 upper layers of the epidermis eventually forming the cornified envelope (Watt et al., 1988).

Many genes encoding major proteins involved in terminal differentiation, such as Filaggrin and Loricrin, are located within a 2Mb region on human chromosome 1q21, known as epidermal differentiation complex (EDC). These EDC proteins are derived from at least three families of structurally, functionally and evolutionary related genes (Marenholz et al., 2001; Mischke et al., 1996; Volz et al., 1993; Zhao and Elder, 1997).

Terminal differentiation programme in the epidermis is regulated by a number of transcription factor families and long non-coding RNAs such as AP1, AP2, KLFs, p63, ANCR and TINCR (Eckert et al., 2013; Jaubert et al., 2003; Kouwenhoven et al., 2015; Kretz et al., 2013; Kretz et al., 2012; Truong et al., 2006).

*1.2.1.1. Stratum Basale (*Basal layer*)*

The *stratum basale* is the innermost layer of the epidermis that is adjacent to the dermis (Figure 1.1). It consists of a monolayer of mitotically active keratinocytes that express keratin 5 (K5), keratin 14 (K14) and keratin 15 (K15) (Alam et al., 2011; Bose et al., 2013). Basal keratinocytes are attached to the basement membrane by hemidesmosomes (Tsuruta et al., 2011). K5 and K14 form keratin filaments that maintains the structural integrity of the skin (Bowden et al., 1987).

A small population of slow cycling epidermal stem cells are found in the basal layer and are proposed to give rise to transient amplifying cells, which terminally differentiate to generate the differentiated progeny (Watt, 1998).

Melanocytes which are pigment producing cells are also found in the basal layer. Melanocytes produce melanin pigment which is transferred into surrounding keratinocytes via melanosomes. Melanin is responsible for skin colouration and protects epidermal cells from harmful effects of UV radiation (Costin and Hearing, 2007; Singh et al., 2010; Tarafder et al., 2014).

Also found in the basal layer are Merkel cells, which are neuroendocrine cells involved in mechanoreception (Boulais and Misery, 2007).

As keratinocytes withdraw from the cell cycle and commit to terminal differentiation, they remain transcriptionally active and move towards the surface of the skin from the stratum *basale* to the *stratum spinosum.*

*1.2.1.2. Stratum Spinosum (*Spinous layer*)*

The *stratum spinosum* lies immediately above the *stratum basale* and consists of several layers of cells (Figure 1.1). As keratinocytes enter the spinous layer, they switch from expressing K5 and K14 to keratin 1(K1) and keratin 10 (K10). K1 and K10 form dense keratin filaments that provide inner strength to these cells (Bowden et al., 1987). In addition to the keratins, keratinocytes in this layer start producing involucrin and transglutaminase (Rice and Green, 1979). Desmosomes connect the cells to one another (Moll et al., 2008).

In addition to keratinocytes, Langerhans cells, which are antigen-presenting dendritic cells involved in the skin's adaptive immune responses, are also found in this layer (Romani et al., 2010).

*1.2.1.3. Stratum Granulosum (*Granular layer*)*

Moving from the spinous layer to the surface, keratinocytes flatten, lose their nuclei and cytoplasm, and contain granules of keratohyalin and lamellar bodies (McGrath and Uitto, 2016). The *stratum granulosum* is named for the keratohyalin granules contained in the cells that give them a granular appearance. Keratinocytes in this granular layer produce filaggrin and loricrin (Mehrel et al., 1990; Rice and Green, 1979).

1.2.1.4. Stratum Corneum (Cornified layer)

Stratum corneum is the outermost layer of the epidermis (Figure 1.1). It consists of flattened cells known as corneocytes, which lose their nuclei, cytoplasmic organelles and undergo cornification. Corneocytes are surrounded by a protein envelope formed by covalent crosslinking of soluble proteins precursors such as loricrin, involucrin and SPRRs by the transglutaminases family of enzymes and embedded into a lipid matrix, which protects them from the external environment (Eckert and Rorke, 1989; Eckert et al., 1993; Eckhart et al., 2013; Yoneda et al., 1992). These cells are shed overtime through the process of desquamation and replaced by new cells from the layers below (Proksch et al., 2008). This cellular progression from *stratum basale* to the skin's surface occurs in about 30 days in humans (McGrath and Uitto, 2016).

The epidermis is separated from the dermis by the basement membrane zone (BMZ) (Tsuruta et al., 2011), which consists mainly of collagens such as type IV and type VII collagens; and laminins such as laminin 332 (laminin 5), laminin 311 (laminin 6) and laminin 511 (laminin 10) (McGrath and Uitto, 2016).

1.2.2. Dermis

The dermis is a tough and resilient layer of the skin (Figure 1.1), which protects the body against mechanical injury and helps in thermal regulation (Bensouilah, 2007). Cell types found in the dermis are mainly fibroblasts and other cell types namely mast cells, macrophages, telocytes and lymphocytes (Ceafalan et al., 2012; Costin and Hearing, 2007; Tobin, 2006). Fibroblasts synthesize collagens such as type I and type III collagens, elastin and structural proteoglycans such as versican (Olsen et al., 1989; Zimmermann et al., 1994). Fibroblasts in the dermis are involved in providing elasticity and tensile strength to the skin; and are also involved in wound healing. Telocytes are cells with a small oval-shaped body, from which emerge very long and thin prolongations known as telopodes. These cells are frequently in contact with stem cells, blood vessels, nerve endings and sebaceous glands within the dermis (Ceafalan et al., 2012; Kang et al., 2015) and are involved in regulating these surrounding cells by releasing and exchanging exosomes (Edelstein et al., 2016). Mast cells are specialized cells that play an important role in both adaptive and innate immunity (Benyon, 1989).

In addition, nerve, vascular, lymphatic networks and skin appendages such as hair follicles, sweat and sebaceous glands are located within the dermis.

The dermis is divided into two layers: *stratum papillare and stratum reticulare.*

1.2.2.1. Stratum Papillare

The superficial portion of this layer has ridge-like projections known as dermal papillae, which extend towards the epidermis, containing blood vessels and Meissner's corpuscles, which are specialised mechanosensory end organs responsible for sensitivity to light touch. Meissner's corpuscles are present in glabrous skin such as the palms of the hands (Fleming and Luo, 2013). This layer is characterised by thin, poorly arranged collagen fibres consisting of type I and type III collagens (Sorrell and Caplan, 2004). The papillary dermis nourishes the epidermis through the network of blood vessels found within it and is involved with thermoregulation of the skin.

1.2.2.2. Stratum Reticulare

This layer is thicker and extends from the base of the *stratum papillare* to the hypodermis. It serves as a boundary between the dermis and hypodermis. The reticular dermis has blood vessels and connective tissue that supports the skin. The collagen fibres found in this layer are well organised and consist primarily of type III collagen. Hair follicles extend through the reticular dermis ending in the adipocyte rich hypodermis. Sebaceous glands, sweat glands, nerves are also found in the reticular dermis (Sorrell and Caplan, 2004).

1.2.3 Hypodermis (Subcutis)

The hypodermis is the deepest layer of the skin (Figure 1.1). It is made up of fat tissue that lies below the dermis that insulates the body from cold temperatures and provides shock absorption. The main cell types found in the hypodermis are adipocytes, which are specialised fat cells that store energy as fat. There are two major types of adipocytes in mammals, white adipocytes, which are involved in storage of fat and brown adipocytes, which are involved in thermoregulation (Algire et al., 2013; McGrath and Uitto, 2016).

1.3. The Hair Follicle

The hair follicle (HF) in mammals is involved in thermal insulation, physical protection, camouflage, excretion, sensation, social interaction, wound repair, dispersion of sweat and sebum (Ito et al., 2005; Schneider et al., 2009). In addition, it also provides a protective niche for epithelial and melanocyte stem cells (Alonso and Fuchs, 2003; Nishimura, 2011; Yang and Cotsarelis, 2010).

1.3.1. Hair Follicle Anatomy

The hair follicle (HF) is a complex structure that undergoes regeneration and regression through continuous cycles of growth and rest (Alonso and Fuchs, 2006).

The fully developed anagen hair follicle can be divided into three anatomical compartments consisting of the infundibulum, isthmus and the inferior segment. The infundibulum and isthmus are permanent, while the inferior segment renews itself in each hair cycle (Randall and Botchkareva, 2009).

The infundibulum lies above the entry to the sebaceous duct and merges with the interfollicular epithelium (Schneider and Paus, 2014). The isthmus is the short region between the point of attachment of the arrector pili muscle and the entry of the sebaceous gland duct. The isthmus harbours the bulge region, which contains hair follicle stem cells (HFSCs) (Cotsarelis et al., 1990; Geyfman et al., 2014). The inferior segment consists of the suprabulbar area and the hair bulb. The suprabulbar area lies below the isthmus and above the hair bulb. The suprabulbar area comprises of three layers, from outermost to innermost, the outer root sheath, inner root sheath and hair shaft (Messenger et al., 2016). The hair bulb, contains the hair matrix that consists of actively proliferating keratinocytes and pigment producing melanocytes that surround the flask-shaped dermal papilla, which consists of specialised fibroblast-like cells embedded in an extracellular matrix rich in basement membrane proteins and proteoglycans (Messenger et al., 2016; Paus and Foitzik, 2004; Randall and Botchkareva, 2009). Activated matrix keratinocytes proliferate rapidly, move upwards and

differentiate to give rise to cells of the hair shaft, as well as the concentric layers of the Inner root sheath. Melanocytes transfer pigment into the developing hair keratinocytes to give the hair its colour. The epithelial portion of the hair follicle is separated from the surrounding dermis by a collagenous layer known as the dermal or connective tissue sheath (Figure 1.2).

The outer root sheath extends from the epidermal basal layer and encloses the HF. The outer root sheath forms the bulge region between the insertion of the arrector pili muscle and duct of the sebaceous gland. The bulge has been identified as a reservoir of multipotent stem cells that are label-retaining, slow cycling (Cotsarelis et al., 1990) and can be identified by molecular markers such as K15, K19, CD200 (human), and CD34 (mouse) (Lyle et al., 1998; Michel et al., 1996; Morris et al., 2003; Ohyama et al., 2006; Rittié et al., 2009).

The inner root sheath consists of four layers, namely the cuticle, Huxley's layer, Henle's layer and companion layer (Schneider et al., 2009). The inner root sheath protects the hair shaft. The cells of the cuticle of the inner root sheath partly overlaps with cells of the cuticle of the hair shaft, anchoring the hair shaft tightly to the HF. Inner root sheath cells produce keratins1/10 and trichohyalin that strengthen the inner root sheath to support and mould the growing hair shaft, as well as guide its upward movement (Randall and Botchkareva, 2009).

The Hair shaft consists of the cuticle, cortex and the medulla. The hair shaft cuticle covers the hair. It also protects and maintains the integrity of the hair shaft (Randall and Botchkareva, 2009).

The dermal papilla consists of densely packed specialised fibroblast-like cells of mesenchymal origin and is enclosed by the hair bulb. It determines the size of the hair bulb, diameter and length of the hair shaft and the duration of anagen (Krause and Foitzik, 2006; Schneider et al., 2009).

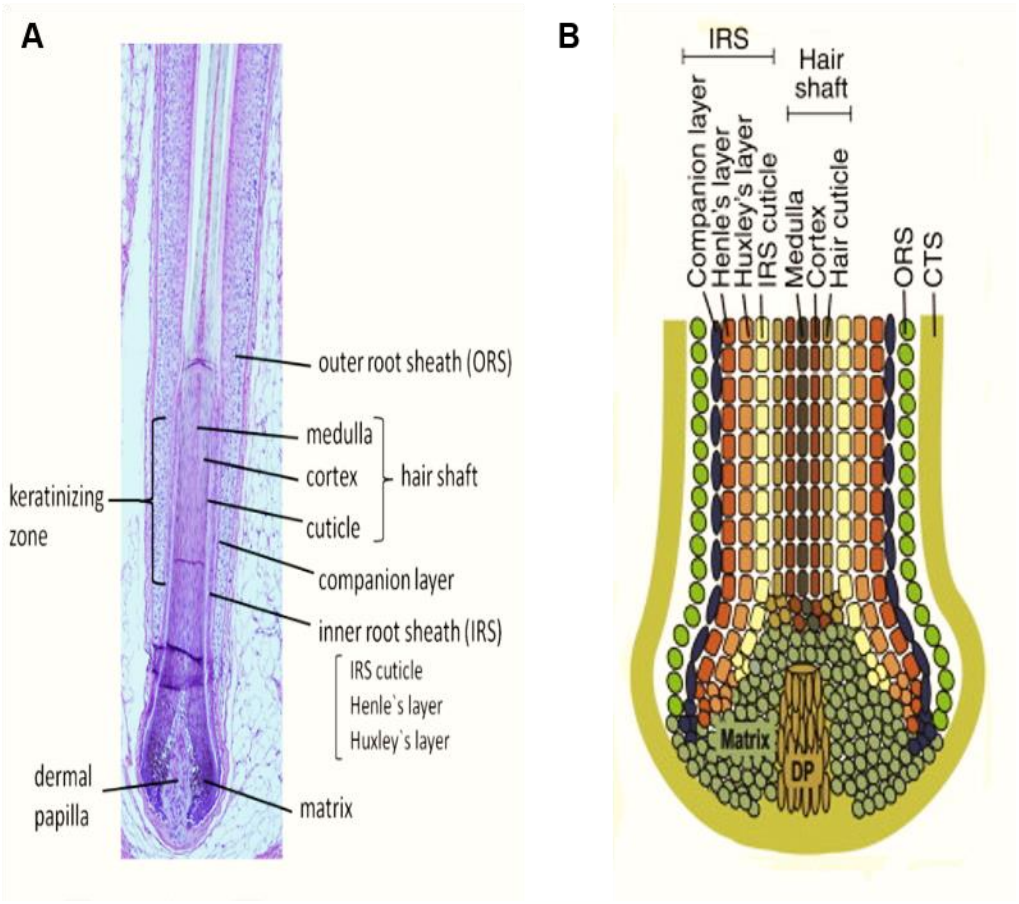


Figure 1.2 The Hair Follicle. (A) Vertical section of human anagen hair follicle (Shimomura, 2013) (B) Schematic drawing illustrating the concentric layers of the outer root sheath (ORS), inner root sheath (IRS) and hair shaft in the bulb. The inner root sheath is composed of four layers: Companion layer (CL), Henle's layer, Huxley's layer, and the inner root sheath cuticle (DP: dermal papilla; IRS: inner root sheath; ORS: outer root sheath; CTS: connective tissue sheath). Adapted from (Schneider et al., 2009).

1.4. Hair Cycle

Hair follicles undergo rhythmic changes through a growth phase known as anagen, a regressive phase known as catagen and a rest phase known as telogen (Figure 1.3). Although hair follicle cycling is regulated by numerous growth factors, cytokines, hormones and signalling pathways, some of which are produced by the hair follicle itself, it is an autonomous phenomenon that continues even in isolated hair follicles in organ culture (Langan et al., 2015; Schneider et al., 2009; Stenn and Paus, 2001).

1.4.1. Anagen

This is the growing phase of the hair follicle and is divided into 6 stages based on specific morphological criteria. During early phases, transient amplifying cells derived from the epithelial hair follicle stem cells in the bulge proliferate rapidly, envelop the dermal papilla, grow downwards into the dermis and differentiate to form the hair shaft and inner root sheath. In mid anagen, hair follicle melanocytes located in the hair matrix show pigment-producing activity, and the newly synthesized hair shaft begins to develop. The synthesis of new hair shaft and pigmentation only occurs during anagen (Krause and Foitzik, 2006). In late anagen, full restoration of the hair fibre-producing unit is achieved, which is characterised by the formation of the epithelial hair bulb surrounding the dermal papilla, located deep in the subcutaneous tissue, and the newly formed hair shaft emerges from the skin's surface (Krause and Foitzik, 2006; Müller-Röver et al., 2001; Paus and Cotsarelis, 1999; Randall and Botchkareva, 2009; Stenn and Paus, 2001). In

humans, almost 85 – 90% of scalp hairs are in this phase. Anagen can last up to two to six years in human scalp hair follicles (Buffoli et al., 2014).

Epidermal-mesenchymal interactions are fundamental drivers of the hair cycle. During anagen, active signal exchanges occur between the epithelial cells of the hair bulb and the fibroblasts of the dermal papilla. Actively proliferating and post mitotic keratinocytes of the hair matrix express receptors and/or intracellular signalling components of a variety of signalling pathways, such as c-kit, c-met, FGFR2 and IGF-IR, while the corresponding ligands, such as SCF, HGF, FGF7, IGF-1 and STAT5 are expressed in the dermal papilla (Danilenko et al., 1995; Itami et al., 1995; Legrand et al., 2016; Lindner et al., 2000). In addition, signalling components of Bone Morphogenetic proteins (BMPs), Wingless (WNT), Fibroblast growth factor (FGF), Sonic Hedgehog (SHH) and Transforming growth factor-beta (TGF-β) have been identified to regulate anagen induction and maintenance (Botchkarev et al., 2001; Greco et al., 2009; Myung et al., 2013; Plikus, 2012).

1.4.2. Catagen

At the onset of catagen, proliferation and differentiation of hair matrix keratinocytes reduces dramatically, melanin production by melanocytes ceases, and hair shaft production is completed. During catagen, the hair matrix and dermal papilla involved in hair production are reduced to sizes that allow them to regenerate in the next hair cycle after receiving the appropriate stimulation. The hair follicle shortens in length by up to 70%.

Morphologically and functionally, catagen phase can be divided into eight sub-stages (Müller-Röver et al., 2001). During catagen, a specialised structure, the club hair is formed (Alonso and Fuchs, 2006; Schneider et al., 2009). The keratinised brush-like structure at the base of the club hair is surrounded by epithelial cells of the outer root sheath and anchors the hair in the telogen follicle. During catagen, the dermal papilla is transformed into a cluster of quiescent cells that are closely adjacent to the regressing hair follicle epithelium and travels from the subcutis to the dermis/subcutis border to maintain contact with the distal portion of the hair follicle epithelium including the secondary hair germ and bulge. Catagen is characterised by several simultaneously occurring and tightly coordinated cellular programs (Randall and Botchkareva, 2009). The most important characteristic feature is a well-coordinated apoptosis occurring in the proximal part of the hair follicle. Apoptosis is regulated differently in each follicle compartment and distinct cell populations show different abilities to undergo apoptosis (Botchkareva et al., 2006). The majority of the follicular epithelial cells and melanocytes are very susceptible to apoptosis, while dermal papilla fibroblasts and the populations of keratinocytes and melanocytes selected for survival display a high resistance (Lindner et al., 1997).

Catagen induction is modulated by various signalling components of Bone Morphogenetic proteins (BMPs), Fibroblast growth factor (FGF), and Transforming growth factor-beta (TGF-β) (Foitzik et al., 2000; Tsuji et al., 2003).

At the end of this regression stage, the hair follicle enters telogen.

1.4.3 Telogen

Telogen hair follicles are very short in length. They are characterised by a lack of pigment-producing melanocytes and the inner root sheath. Their compact ball shaped dermal papilla is closely attached to a small cap of secondary hair germ keratinocytes containing hair follicle stem cells (Randall and Botchkareva, 2009). During telogen, the hair shaft matures into a club hair that is shed from the follicle (Paus and Cotsarelis, 1999). This resting phase typically lasts for two to three months before hair follicles of the human scalp re-enter anagen and the cycle is repeated. Telogen to anagen transition occurs at the activation of bulge stem cells to proliferate and form the new hair shaft.

1.4.4 Exogen

An additional phase of the hair cycle known as exogen involves the shedding of the hair shaft from the telogen follicle. It is an active process that is accompanied by the activation of proteolytic processes in the follicular root (Milner et al., 2002). Exogen is independent of telogen and anagen. Van Neste et al. showed that in human hair follicles, anagen and telogen hairs are firmly anchored to the follicle, while exogen hairs are passively retained within the follicles. In addition, exogen clubs do not retain remnants of the outer root sheath, in contrast to plucked telogen hairs (Van Neste et al., 2007).

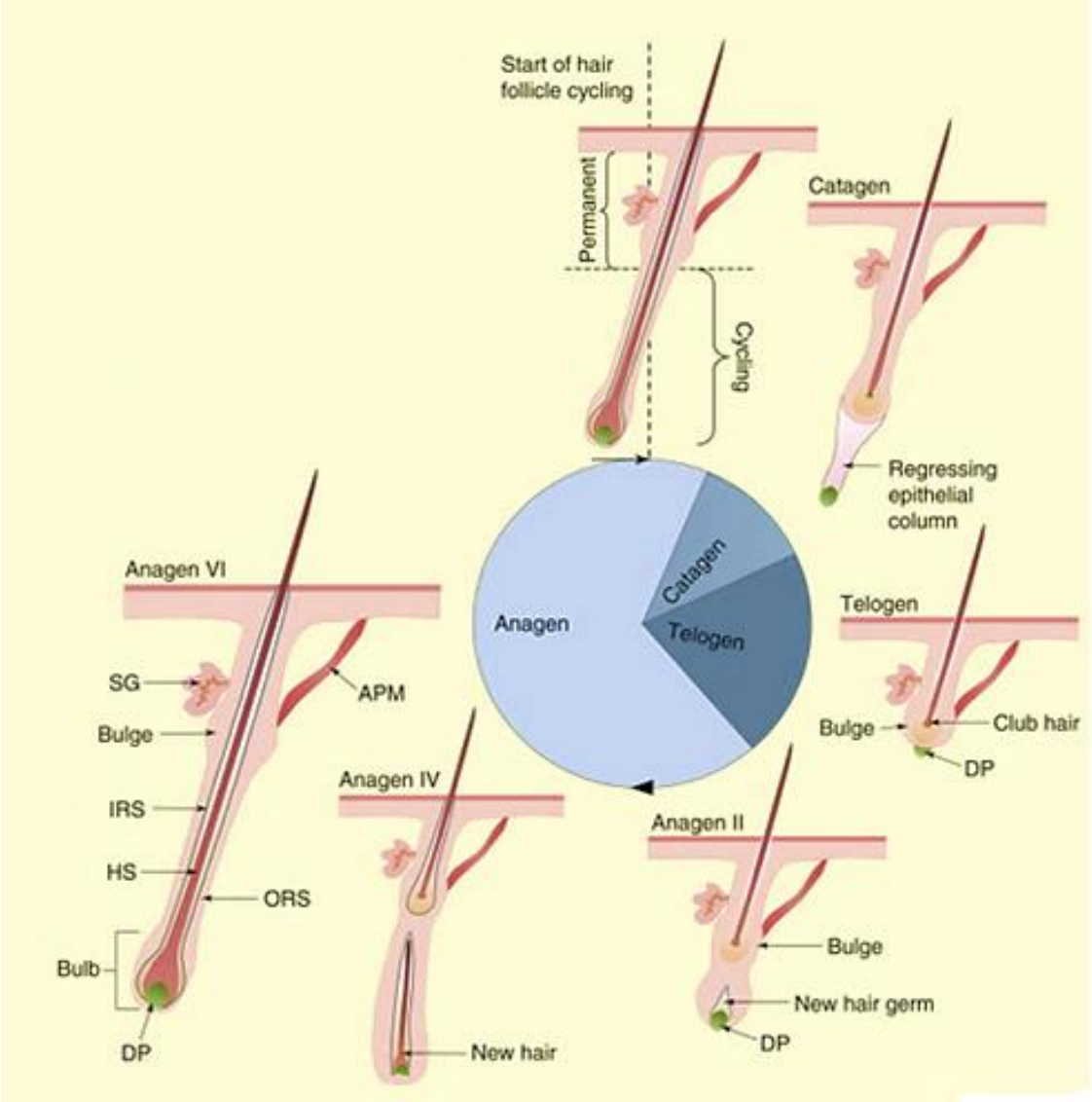


Figure 1.3 – **Key stages of the hair cycle**. The hair cycle is divided into three phases: anagen (growth phase), catagen (regression phase) and telogen (resting phase). APM: arrector pili muscle; DC, dermal condensate (green); DP: dermal papilla (green); HS: hair shaft (brown); IRS: inner root sheath (blue); MC: melanocytes; ORS: outer root sheath; SC: sebocytes (yellow); SG: sebaceous gland. Adapted from (Schneider et al., 2009).

1.5. Epigenetic mechanisms controlling skin and hair follicle development

Epigenetics studies the stable and heritable alterations in gene expression that occur without alterations in DNA sequence (Berger et al., 2009; Jaenisch and Bird, 2003).

Increasing evidence suggests that epigenetic modifications play crucial roles in mammalian skin development (Dauber et al., 2016; Driskell et al., 2011; Ezhkova et al., 2011; Sen et al., 2010; Sen et al., 2008). These modifications include DNA modifications, post translational histone modifications and Chromatin remodelling.

1.5.1. DNA Modification

DNA modification processes that control gene expression include DNA methylation and hydroxylmethylation.

1.5.1.1. DNA Methylation

DNA methylation has been studied extensively. It involves the addition of a methyl group from S-Adenosyl-methionine (SAM) at the 5' position of a cytosine within the genome by a family of enzymes known as DNA Methyltransferases (DNMTs) to form 5-methylcytosine (Feng et al., 2010).

DNA methylation is present in about 4-8% of the cytosine bases within the human genome, depending on the cell type (Lister and Ecker, 2009; Lister et al., 2009). Most DNA methylation occurs within CpG dinucleotides but it has been reported to occur outside the CpG context in human DNA (Lister et al., 2009; Yan et al., 2011). Within the human genome, about 30 million CpG dinucleotides exist either in a methylated or an un-methylated state (Cocozza et al., 2011).

DNA methyltransferases include DNMT1, DNMT3A, DNMT3B and DNMT3L. DNMTs are essential for establishing and maintaining DNA methylation patterns. During cellular replication, DNA methylation patterns are passed from parent to daughter cells. DNMT1 ensures that programmed DNA methylation patterns remain through cellular generations and is referred to as maintenance DNMT (Lei et al., 1996; Li et al., 1992). DNMT3A and DNMT3B catalyse the methylation of both unmodified cytosines and hemi-methylated cytosines producing new DNA methylation marks. They are referred to as *de novo* DNMTs (Okano et al., 1999). DNMT3L lacks catalytic activity and acts as a cofactor for both DNMT3A and DNMT3B (Goll and Bestor, 2005). DNA methylation is usually associated with gene repression but it has been demonstrated that it can also contribute to gene activation (Rishi et al., 2010). DNA methylation represses gene expression either through inhibiting the recognition of DNA by transcription factors or facilitating the binding of methyl-CpG binding proteins to DNA (Klose and Bird, 2006). DNA methylation can contribute to gene activation through the creation of new binding sites on target genes for the recruitment of the transcription factor C/EBPα (Rishi et al., 2010).

DNMT1 is expressed in the basal layer, hair follicle ORS and matrix cells in human and mice. In epidermal tissues, DNA methylation patterns are essential for the maintenance of progenitor cells and tissue renewal (Sen et al., 2010). DNMT1 and UHRF1, a protein which form complexes with DNMT1 and recruits it to hemi-methylated DNA (Bostick et al., 2007; Sharif et al., 2007), are expressed in the basal layer of the epidermis and are down regulated during differentiation. Depletion of DNMT1 in human primary keratinocytes resulted in progressive tissue loss and premature differentiation (Sen et al., 2010). Epidermal deletion of DNMT1 in mice led to hyper-proliferation in the epidermis and progressive hair loss associated with increased $p16^{INK4A}$ expression and a prolonged telogen phase (Li et al., 2012).

1.5.1.2. DNA Hydroxymethylation

For a long time, DNA methylation marks were thought to be stable but the removal of the methyl group from cytosine base has been reported. This epigenetic mark known as DNA hydroxymethylation shows that DNA methylation is reversible. The ten-eleven translocation (TET) protein family of enzymes consists of TET1, TET2 and TET3. TET protein family of enzymes catalyse the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in an iron (II), α-ketoglutarate and divalent oxygen dependent manner (Ito et al., 2010; Tahiliani et al., 2009). In addition, TET proteins also catalyse the demethylation of 5mC by the further oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), followed

by TDG-dependent base excision repair to unmodified cytosine (Figure 1.4) (He et al., 2011; Ito et al., 2011; Wu and Zhang, 2017).

The role of DNA hydroxymethylation in mammalian skin development is unclear.

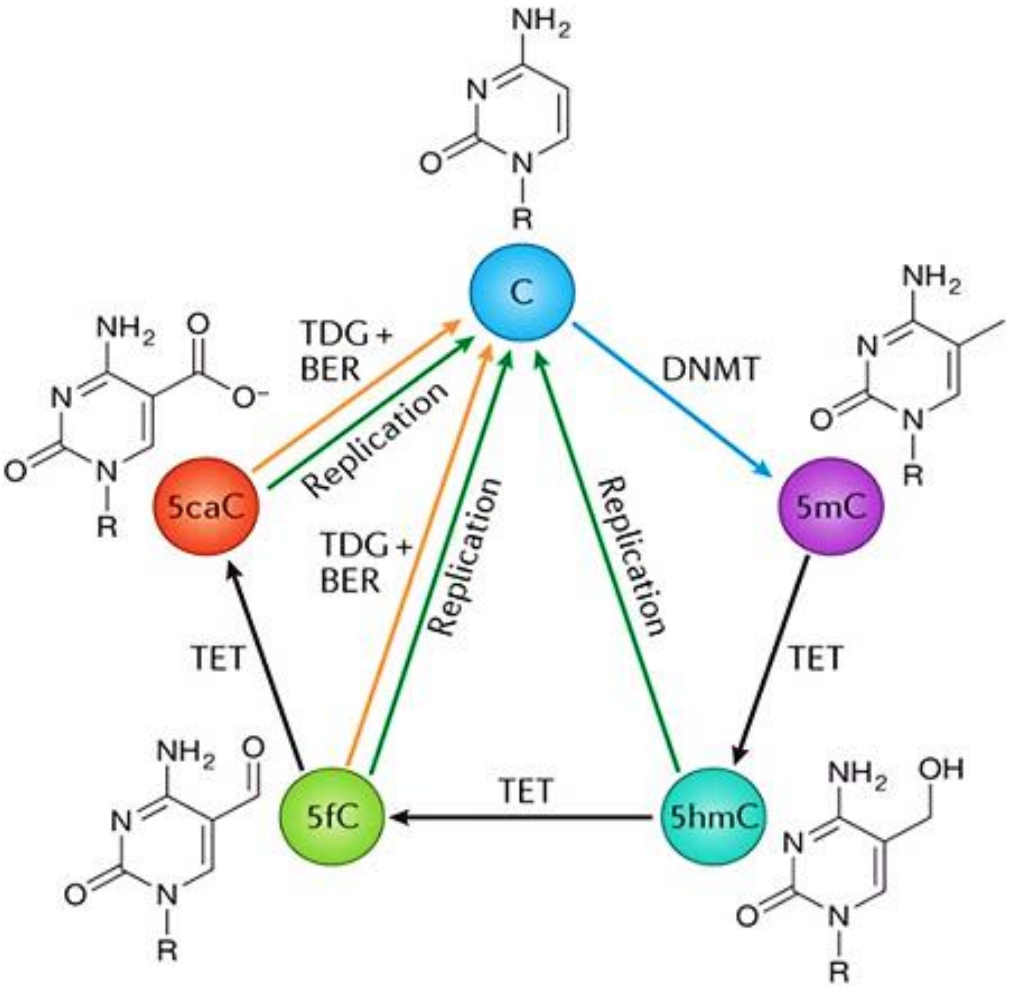


Figure 1.4 The cycle of active DNA demethylation. DNA methyltransferases (DNMTs) convert unmodified cytosine to 5-methylcytosine (5mC). 5mC can be converted back to unmodified cytosine by TET-mediated oxidation to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), followed by excision of 5fC or 5caC mediated by thymine DNA glycosylase (TDG) coupled with base excision repair (BER). Adapted from (Wu and Zhang, 2017)

1.5.2. Histone Modifications

In mammals, genomic information is organised into chromatin. The basic unit of chromatin is the nucleosome which consists of 147bp of DNA wrapped around an octamer of two molecules each of four core histone molecules – H2A, H2B, H3 AND H4 (Margueron and Reinberg, 2010).

These core histone molecules are subject to a variety of post-translational modifications (PTMs) mostly in their N-terminal tails. These histone PTMs include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, arginine deamination and proline isomerisation (Berger, 2002; Botchkarev et al., 2012; Kouzarides, 2007). Histone PTMs have been identified as modifications involved in epidermal development.

1.5.2.1. Histone Acetylation

Histone acetylation involves the addition of an acetyl group from acetyl-coenzyme A onto the ε-amino group of lysine catalysed by histone acetyltransferases (HATs). Histone acetylation neutralises the positive charge of lysine and this in turn weakens the interaction between the histone tail and DNA (Allfrey et al., 1964). In mammals, HATs are diverse and are grouped into at least 5 different families including

1. HAT1
2. GNAT family which includes Gcn5 and PCAF
3. MYST family including members such as Tip60
4. P300/CBP family which includes p300 and CBP

5. Rtt109 (Marmorstein and Trievel, 2009; Schneider et al., 2013).

Histone acetylation is reversible. Histone deacetylases (HDACs) catalyse the removal of the acetyl group from lysine residue. In mammals, 18 HDACs have been characterized and grouped into the following 4 major classes:

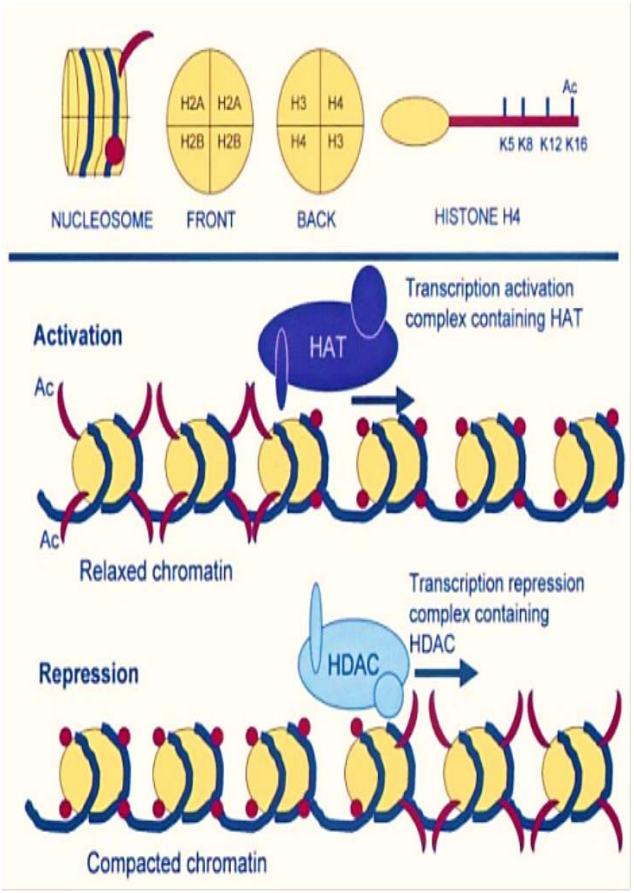
1. Class I HDACs (HDAC 1, 2, 3 and 8) are localised in the nucleus and are expressed ubiquitously in all cells.
2. Class II HDACs (HDAC 4, 5, 6, 7, 9 and 10) are localised mainly in the cytoplasm but can also shuttle between the cytoplasm and the nucleus when needed. Their expression is cell-type specific. They are further divided into class IIa (HDAC 4, 5, 7 and 9) and class IIb (HDAC 6 and 10).
3. Class III HDACs include the nicotinamide adenine dinucleotide (+)-dependent (NAD^+-dependent) Sirtuins – SIRT 1 – 7. SIRT 1, 6, and 7 are localised in the nucleus. SIRT2 is localised in the cytoplasm while SIRT3, 4 and 5 are localised in the mitochondria (Michishita et al., 2005).
4. Class IV contains only one member – HDAC 11 which is localised in the nucleus (de Ruijter et al., 2003).

HATs catalyse the addition of acetylation marks to histone leading to open chromatin and transcriptional activation whereas HDACs catalyse the removal of acetylation marks from histone leading to compact chromatin and transcriptional repression (Shahbazian and Grunstein, 2007)(Figure 1.5).

The promoter region of active genes is enriched in H3K9ac, H3K18ac and H2B12ac while the coding region of the active gene is enriched in H4K12ac

and H4K16ac (Botchkarev et al., 2012; Wang et al., 2009). Histone acetylation levels are significant in keratinocyte-specific gene expression programs. For example, treatment of primary human keratinocytes with HDAC inhibitors - TSA and sodium butyrate induced growth arrest via reduction of the expression of the proliferation gene *CDK1* and induction of the expression of differentiation markers involucrin and transglutaminase 1 (Elder and Zhao, 2002; Saunders et al., 1999). Conditional deletion of HDAC1 and HDAC2 in embryonic mouse skin resulted in defective proliferation and stratification in the epidermis and no hair follicle development with an increase in apoptosis via the regulation of p63 and p53 (LeBoeuf et al., 2010). Consistent with this data, ablation of HDAC 1 and HDAC2 in adult mouse skin led to severe epidermal defects, disruption in hair follicle regeneration and homeostasis, abnormal pigmentation and spontaneous tumour formation with impaired Sin3A co-repressor complex function, increased level of c-Myc and p53 expression (Hughes et al., 2014; Winter et al., 2013). Also ablation of HDAC1 in a skin tumour model led to accelerated tumour development suggesting a tumour suppressor role for HDAC1 in skin (Winter et al., 2013). In human keratinocytes, SIRT1 inhibits proliferation and promotes differentiation *in vitro* (Blander et al., 2009).

HAT
- HAT1
- P300/CBP family (p300, CBP)
- GNAT family (GCN5, PCAF, ELP3)
- MYST family (TIP60, MYST1-4)
- Rtt109



HDAC Class:
- I: HDAC1, 2, 3, 8
- IIa: HDAC4, 5, 7, 9
- IIb: HDAC6, 10
- III: SIRT1-7
- IV: HDAC11

Figure 1.5 – **Histone acetylation.** A diagram showing the dynamics between transcriptional activation by HATs and transcriptional repression by HDACs. Adapted from (de Ruijter et al., 2003; Schneider et al., 2013).

1.5.2.2. Histone Methylation

Histone methylation occurs on the side chains of lysines and arginines. Lysines may be mono-, di, or tri-methylated while arginine may be mono- or di-methylated. In addition, arginine can be di-methylated symmetrically or asymmetrically (Bannister and Kouzarides, 2011). Histone methylation is catalysed by histone methyltransferases (HMTs), while demethylation is mediated by demethylases (Wang et al., 2009). Here, lysine methylation is discussed further.

Lysine methylation involves the transfer of a methyl group from S-adenosyl-methionine (SAM) to a lysine's ε-amino group. This transfer is catalysed by a group of lysine methyltransferases (HKMTs) containing the SET domain [Su(var), Enhancer of zeste, Tritotax]. This group of SET domain containing lysine methyltransferases is sub-grouped into 7 families – SUV39, SET1, SET2, EZ, RIZ, SMYD AND SUV4-20 and also includes a few orphaned members –SET7/9 and SET8 (Dillon et al., 2005). In humans, DOT1L, a HKMT that does not contain the SET domain has been identified (Feng et al., 2002; Min et al., 2003a).

Lysine methylation in the H3 molecule could be associated with either transcription activation or repression depending on the degree and site of the modification in the histone tail (Barski et al., 2007). In general, H3K9, H3K27 and H3K64 methylation are associated with transcription repression, while H3K4, H3K36 and H3K79 methylation are associated with transcription activation. For example, the removal of the repressive mark H3K27me3 during epidermal differentiation is mediated by the demethylase JMJD3 (Sen

et al., 2008). In the absence of JMJD3, the expression of differentiation markers such as KRT1, KRT10, IVL and S100A8 were repressed and epidermal differentiation was prevented (Sen et al., 2008). The role of Setd8, a histone methyltransferase that catalyses the mono methylation of histone H4 at lysine 20 (H4K20me) in epidermal development was demonstrated in Setd8 null epidermis (Driskell et al., 2011). Ablation of Setd8 in the epidermis resulted in impaired proliferation and differentiation via the regulation of p63 and p53 expression (Driskell et al., 2011).

1.5.3 Polycomb group proteins

Polycomb group (PcG) proteins, first identified in *Drosophila melanogaster* as crucial regulators of developmental processes, form chromatin remodelling complexes known as Polycomb Repressive Complex (PRC), which silence genes by chromatin compaction (Kennison, 1995). In mammals, they are classified into two groups based on their association with different classes of multimeric protein complexes, Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). The constituents of each complex may differ among organisms and may be cell type dependent, but their core subunits are conserved across organisms. PRC1 complex consists of CBX (CBX2-4-6-7-8), RING1 (RING1A/B), PCGF (PCGF1-6) and HPH (HPH1-3), while the PRC2 complex consists of Enhancer of zeste homolog 1 or 2 (EZH1 or EZH2), Suppressor of zeste 12 (SUZ12) and Embryonic ectoderm development (EED) (Figure 1.6) (Connelly and Dykhuizen, 2017; Di Croce and Helin, 2013). The way these complexes

are recruited to chromatin to impact compaction and gene silencing is still being studied. In addition to the core PRC2 subunits, several accessory proteins have been identified that associate with PRC2. These accessory proteins may enhance PRC2 enzymatic activity and facilitate its recruitment to chromatin. These accessory proteins include RbAp48/p46, AEBP2, JARID2, PHF1, MTF2, PHF19, C17orf96 and C10orf12 (Li et al., 2010; Sarma et al., 2008; Zhang et al., 2011).

PRC2 is the first of the two PRC complexes that is recruited to chromatin, where it catalyses the trimethylation of lysine 27 on Histone 3 (H3K27me3) through the activity of one of its components, EZH1/EZH2 (Cao et al., 2002; Shen et al., 2008), then the CBX component of PRC1 recognises the H3K27me3 histone mark and recruits PRC1 complex which assists in chromatin compaction and gene repression (Min et al., 2003b; Zhang et al., 2012). Once PRC1 is bound to chromatin, it catalyses the mono-ubiquitination of histone H2A at lysine 119 (H2AK119ub1) through the activity of its RING1 component leading to chromatin compaction (Wang et al., 2004). Interestingly, a non-canonical PRC1 without the CBX and HPH components have been reported (Schwartz and Pirrotta, 2014). This has opened the debate on how PRC1 and PRC2 are recruited to chromatin. There is evidence that demonstrates that PRC1-mediated mono-ubiquitination of H2A at lysine 119 leads to the recruitment of PRC2 and H3K27 tri-methylation at an unmethylated CpG island (Blackledge et al., 2014; Cooper et al., 2014). Another study also reports the recruitment of PRC2 by PRC1-mediated H2AK119ub1, but this was via the interaction of

PRC2 complexes containing AEBP2 and JARID2 with the H2AK119ub1 mark (Kalb et al., 2014).

These Polycomb protein complexes are crucial in the control of tissue development, stem cell maintenance, cell cycle progression, senescence, X chromosome inactivation and cancer (Bracken et al., 2007; Casanova et al., 2011; Richly et al., 2011; Simon and Kingston, 2009).

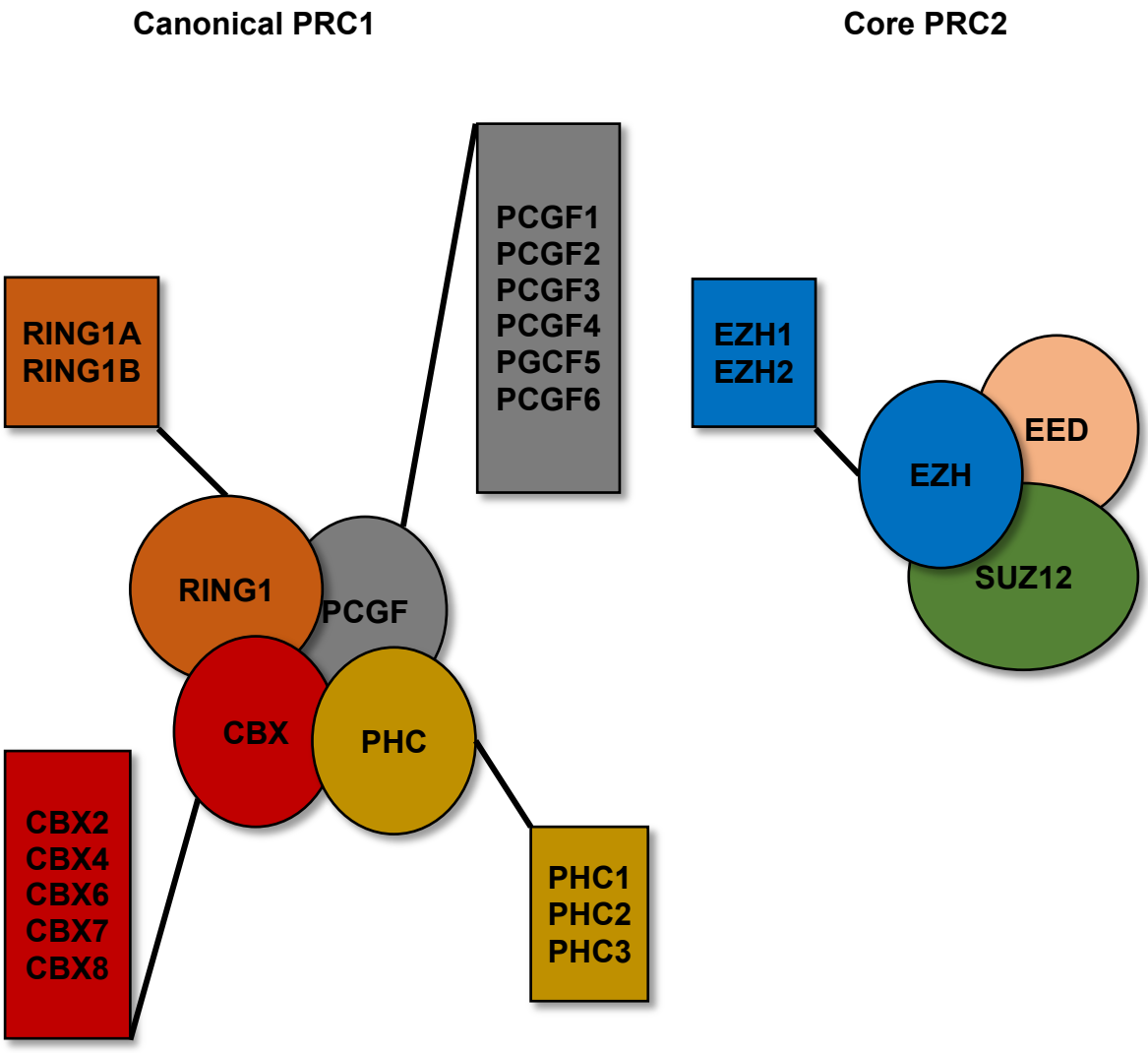


Figure 1.6 - Composition and functions of the main Polycomb Repressive Complexes, PRC1 and PRC2. Adapted from (Connelly and Dykhuizen, 2017; Di Croce and Helin, 2013).

1.5.4 Polycomb Repressive Complexes in mammalian skin development and homeostasis

Polycomb repressive complexes play important roles during skin development and homeostasis. In the human epidermis, PRC1 component CBX4 regulates proliferation and differentiation of epidermal progenitor cells while protecting them from senescence. It is therefore required for epidermal progenitor cell maintenance (Luis et al., 2011). CBX4 exerts its function on proliferation and differentiation via its E3-SUMO ligase activity and represses senescence in a PRC1-dependant manner via its chromodomain (Luis et al., 2011). In mouse skin, Cbx4 also maintains epidermal progenitor cell identity and controls proliferation of epidermal keratinocytes via the regulation of non-epidermal lineage and cell-cycle inhibitor genes (Mardaryev et al., 2016).

Another PRC1 component BMI-1, also known as PCGF4, is expressed in the basal and suprabasal layers of human epidermis (Lee et al., 2008; Reinisch et al., 2007; Ressler et al., 2006). BMI-1 promotes keratinocyte proliferation and survival by increasing the expression of cyclin D1; and protects keratinocytes from apoptosis mediated by stress agents via regulating caspase activation and PARP cleavage (Eckert et al., 2011; Lee et al., 2008). BMI-1 also controls keratinocyte senescence by regulating the INK4a/ARF locus (Cordisco et al., 2010; Ressler et al., 2006; Silva et al., 2006).

The core components of PRC2 are expressed in the epidermis. Studies in PRC2-null mouse epidermis shows that loss of PRC2 in epidermal

progenitors and hair follicle progenitors give rise to distinct skin phenotypes in the epidermis and HFs. In the epidermis, loss of PRC2 enzymatic activity (Ezh1/2-null) in epidermal progenitors resulted in accelerated epidermal barrier formation, epidermal hyper proliferation and promoted their differentiation to Merkel cells via the regulation of Sox2 (Bardot et al., 2013; Dauber et al., 2016; Ezhkova et al., 2009; Perdigoto et al., 2016) while in HF progenitors, loss of PRC2 resulted in an upregulation of the Ink4a/Ink4b/Arf locus genes leading to a reduction in HFSC proliferation and eventually degeneration of the hair follicles as a result of increased apoptosis (Dauber et al., 2016; Ezhkova et al., 2011).

Jarid2, an accessory member of PRC2 is reported to be necessary for epidermal homeostasis in mouse. Mejetta et al reported that in mouse skin, HF stem and progenitor cells require Jarid2 for their activation, proliferation and differentiation (Mejetta et al., 2011).

1.5.5 Polycomb Repressive Complexes in skin diseases and cancer

The role of PRC subunits in the pathogenesis of skin diseases such as psoriasis and atopic dermatitis is not clear but increasing evidence suggests that they may be involved in the pathogenesis of these diseases. For example, EZH2 has been reported to be elevated in lesional psoriatic skin and Liu et al. speculated that overexpression of EZH2 in psoriatic skin blocks AP1 activity and prevents effective differentiation of epidermal cells (Liu et

al., 2011). Understanding the role of PRC2 subunit in the pathogenesis and progression of skin diseases remains to be elucidated.

Skin cancer is among the most common cancers in the world. Skin cancers include melanoma and non-melanoma skin cancers, which are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Increasing evidence suggests a role for PRC subunits in the development and progression of skin cancers. However, the mechanisms by which PRC subunits regulate skin tumorigenesis are unclear and the focus of ongoing research. Several PRC subunits have been reported to be upregulated in skin cancers. Analysis of skin tumours cells, cell lines and genetic mouse models have revealed overexpression of BMI-1 and EZH2 in SCC, BCC and melanoma (Adhikary et al., 2015; Adhikary et al., 2013; Lee et al., 2008; Mihic-Probst et al., 2007; Reinisch et al., 2007; Zingg et al., 2015), while SUZ12 is overexpressed in melanoma and BCC (Martín-Pérez et al., 2010). Interestingly, tumour initiating cells known as cancer stem cells, which were enriched for BMI-1, EZH2 and H3K27me3, have been identified in SCC (Adhikary et al., 2013). These skin cancer stem cells require EZH2 for their survival, migration, invasion and tumour formation (Adhikary et al., 2015). Zingg et al. demonstrated a role for EZH2 in promoting melanoma growth and progression using a genetic mouse model (Zingg et al., 2015).

The elevated levels of PRC subunits in various solid tumours, including skin cancers, has led to the proposal that modulating their activities can be employed as a strategy for suppressing tumour formation. For example, in skin cancer cell lines, treatment with sulforaphane, DZNep and EGCG reduced the expression of EZH2 resulting in growth arrest and increased

apoptosis (Balasubramanian et al., 2010; Choudhury et al., 2011). Treatment of SCC-13 cells with small molecule inhibitors, such as GSK126, resulted in reduced tumour formation when injected in NSG mice (Adhikary et al., 2015).

Interestingly, these commercially available small molecule inhibitors of PRC subunits can be employed as an approach in depleting PRC subunits activity *in vivo and in vitro* to study their distinct functional role in skin development, homeostasis, cancer and diseases. A better understanding of the role of PRC subunits in human skin development, homeostasis and cutaneous disorders could lead to the possible use of these small molecule inhibitors as a therapeutic strategy for modulating PRC activities in skin cancer and skin diseases in the future.

1.6 Aims and Objectives

Progress over the past decade using conditional skin KO mice as a model has clearly defined a role for PRC2 in epidermal stem cell maintenance, epidermal homeostasis and HF homeostasis. However, a role for PRC2 in human epidermal homeostasis and HF regeneration remains largely unclear.

The aim of this project is to determine the role of PRC2 in human skin homeostasis and HF regeneration. This will be addressed by the following aims:

1. Investigate the expression of a PRC2 subunit – EZH2 in human skin tissue, epidermal cells sorted from human epidermis, hair follicles and hair follicle stem cells by employing Immunofluorescence, cell culture, RT-qPCR and Western blot.

2. To determine the target genes of EZH2 using RNAi approach and Microarray analysis

3. To determine the functional role of PRC2 in regulating gene expression during terminal differentiation in human epidermis by modulating its activity utilising selective small molecule inhibitors or RNAi approaches.

4. To evaluate the effect of modulating EZH2 activities using selective small molecule inhibitors or RNAi approaches on human hair growth *ex vivo* and primary hair follicle keratinocytes *in vitro.*

CHAPTER 2: Materials and Methods

2.1 Ethics

Keratinocytes and hair follicles were isolated and cultured from skin tissue from individuals undergoing cosmetic surgery (face lift) and hair transplant. Patients gave informed written consent and ethical approval was granted by the University of Bradford ethics committee. Skin tissue was prepared on the day of collection.

2.2 Cell Culture

2.2.1 Isolation of keratinocytes from adult skin

Skin tissue specimen was obtained from facelift patients aged between 55 – 67 years after undergoing cosmetic surgery (details in table 2.1). Skin samples were stripped of fat using scissors or razor blades and were cut up into approximately 1.0 cm^2 pieces. Cut skin samples were thoroughly washed in PBS. Skin was incubated overnight submerged (epidermis side up) in 0.25% Trypsin at 4°C and then heated to 37°C for 30 minutes prior to removal of the epidermis. The epidermis was removed and incubated for 5 minutes at 37°C in a water bath in 1 ml 0.05% Trypsin/0.02%EDTA (TE) and vortexed for 1 minute thereafter. The resulting trypsinised cell suspension was carefully aspirated and transferred into serum-containing media to neutralise TE. Incubation in TE was repeated twice more. Cells-containing suspension was centrifuged for 5 minutes at 1000 RPM to pellet cells. The

cell pellet was re-suspended in KBM2/DMEM (ratio 2:1) and cells were seeded in T75 flask. DMEM (Gibco) supplemented with sterile 10% Fetal bovine serum (Gibco), 25mM HEPES (Gibco), 100 units/ml penicillin and 100µg/ml streptomycin (Gibco), 2mM L-glutamine (Gibco) and 100mM sodium pyruvate (Gibco). Media was replaced with KBM2 medium (PromoCell) supplemented with 0.004ml/ml Bovine Pituitary Extract, 0.125ng/ml recombinant human Epidermal Growth Factor, 5µg/ml recombinant human insulin, 0.33µg/ml hydrocortisone, 0.39µg/ml epinephrine, 10µg/ml transferrin and 0.06mM $CaCl_2$ (PromoCell); two days after initial seeding and cells were fed every 2-3 days until sub-confluent.

2.2.2 Isolation of keratinocytes from human hair follicles

Hair Follicles obtained from hair transplant surgery (details in table 2.1) with the top and dermal papilla cut off were put in DMEM (Gibco) supplemented with sterile 10% Foetal bovine serum (Gibco), 25mM HEPES (Gibco), 100 units/ml penicillin and 100µg/ml streptomycin (Gibco), 2mM L-glutamine (Gibco) and 100mM sodium pyruvate (Gibco). Media was replaced with KBM2 medium (PromoCell) supplemented with 0.004ml/ml Bovine Pituitary Extract, 0.125ng/ml recombinant human Epidermal Growth Factor, 5µg/ml recombinant human insulin, 0.33µg/ml hydrocortisone, 0.39µg/ml epinephrine, 10µg/ml transferrin and 0.06mM $CaCl_2$ (PromoCell); two days after initial seeding and cells were fed every 2-3 days until subconfluent.

2.2.3 Culturing and passaging of keratinocytes

Cells in culture flasks isolated from adult skin, human hair follicles and juvenile foreskin obtained from Promocell (Promocell) were usually split when they reached between 80 and 90% confluence. Cells were washed under the safety cabinet with 5ml pre-warmed DPBS (Gibco). DPBS was removed and 5ml 0.5mM EDTA added flask was transferred to the incubator for 5 minutes to break cell-cell interaction. At this stage, the cells have become single cells and round. EDTA was removed and 3ml 0.05% Trypsin/0.02%EDTA (TE) added as above but for about 3 minutes. TNS (PromoCell GmbH) was added to cells to inactivate the activity of trypsin. Amount added was equal to the volume of TE. Cells were then transferred into 15 ml Falcon tube and centrifuged at 1500 RPM for 3 minutes. Cell pellet was either re-suspended in KBM2 medium or frozen as described below. Following resuspension in KBM2 medium, cells were counted immediately using Haemocytometer chamber and seeded for experiments or further culture.

For analysing EZH1, EZH2 and cell cycle regulators expression in aged and young NHEKs, NHEKs pooled from adult keratinocytes isolated from female individuals aged 57 and 64 (Aged) and isolated from juvenile foreskin from a single donor (Young) (Promocell) were seeded at 2×10^5 cells and grown till 60 -70% confluent. Media was changed to KBM2 medium without supplements for 24hrs for cell cycle synchronisation and switched back to complete KBM2 medium before treatment with EZH1/2 and EZH2 inhibitors. Cells were collected at day 3 or 4 after treatment for total RNA isolation.

2.2.4 Freezing and Thawing of keratinocytes

Cell suspension obtained in the context of cell passaging was transferred to a 15ml Falcon tube and centrifuged at 1000 RPM for 3 minutes. Supernatant was discarded, and cell pellet was re-suspended in 1ml CryoSFM freezing medium (Promocell, UK) per 1-2 $x10^6$ cells. Cells were transferred into the freezing vial(s). The vial(s) was transferred into Mr. Frosty which was taken to the -80^oC freezer overnight before cells were transferred into the liquid nitrogen (-196^oC) for storage until needed.

To thaw cells, vials containing the cells were left in 37^oC water bath until almost completely thawed. Cells were transferred into already pre-warmed complete KBM2 medium in T75 culture flask (Corning Life Sciences) and the flask transferred into the incubator at 37^oC, 5% CO_2 for the cells to attach to the flask and to continue proliferating until ready for the next passage.

2.3 Isolation of human hair follicles

Scalp tissue specimen was obtained from facelift and hair transplant patients aged between 45 – 69 years after undergoing cosmetic surgery (details in table 2.1). Tissue samples containing more than 50 hair follicles were dissected into single hair follicles using surgical blade and forceps under a stereo dissecting microscope (Nikon, USA). Hair follicles considered to be in the anagen stage morphologically were used for hair follicle organ culture.

2.4 Hair Follicle organ culture

Human scalp hair follicles were isolated and cultured in vitro, as described previously (Philpott et al., 1990). Briefly, dissected hair follicles were cut into small pieces of approximately 2.2mm in length from the bottom of dermal papillae and cultured in 24-well cell culture plates for 6 days in William's E medium (Gibco) supplemented with 10µg/ml hydrocortisone (Sigma), 10µg/ml insulin, 2mM L-glutamine (Gibco), 1µg/ml streptomycin and 1000units/ml penicillin (Gibco) at 37°C in a 5% CO_2 atmosphere. HFs were either transfected with siRNA targeted at EZH2 or treated with a small molecule inhibitor of EZH2 - DZNep. Images were taken of hair follicles on day 0 before transfection or treatment, day 3, 6 and 7 post treatment or transfection. Images were captured using a Nikon Eclipse TS100 Light microscope (Nikon Instruments Inc., USA) and Vicicam camera (VWR, UK). Hair follicle length was measured using ImageJ software (NIH Image analysis website http://rsb.info.nih.gov/ij/).

Donor ID	Anatomical Site	Sex	Age	Passage number	Used For
447	Facial skin	Female	57	P3 – P5	Isolation/Culture of keratinocytes
448	Facial skin	Female	64	P3 – P5	Isolation/Culture of keratinocytes
484	Facial skin	Female	65	P3 – P5	Isolation/Culture of keratinocytes
451	Facial skin	Female	67	P3 – P5	Isolation/Culture of keratinocytes
488	Facial skin	Female	64	P3 – P5	Isolation/Culture of keratinocytes
484	Facial skin	Female	65	P3 – P5	Isolation/Culture of keratinocytes
513	Facial skin	Female	55	P3 – P5	Isolation/Culture of keratinocytes
536	Facial skin	Female	57	N/A	IHC
12562	Occipital scalp	Male	55	P3 – P4	Isolation/Culture of ORS keratinocytes
169	Occipital scalp	Male	47	N/A	HFOC
391	Facial skin	Female	52	N/A	HFOC/IHC
360	Occipital scalp	Female	67	N/A	HFOC
368	Occipital scalp	Male	69	N/A	HFOC/IHC
770	Occipital scalp	Male	45	N/A	HFOC
1158	Occipital scalp	Male	55	N/A	HFOC

Table 2.1 Origin of primary cells and hair follicles. Abbreviations used in table: IHC – Immunohistochemistry. HFOC – Hair follicle organ culture. ORS – Outer root sheath. N/A – Not applicable.

2.5 Cell and Hair follicle transfection

NHEKs at 60–70% confluency was transfected with 50nM of siRNAs targeting EZH2 or non-targeting negative control (ON-TARGETplus SMARTpool siRNA – Human, Dharmacon, USA), EED or scrambled negative control (Origene, USA) using Lipofectamine RNAimax reagent (Invitrogen, USA) according to manufacturer's instruction. After 24 - 72 hours, cells were subsequently analysed for gene expression by RT-qPCR and/or Western blot analyses.

For HFs, microdissected human anagen HFs were transfected with a mixture of siRNA against EZH2, provided as a single reagent (Accell SMARTpool – Human EZH2, Dharmacon) in organ culture and with non-targeting siRNA as negative control (Accell Red Non-targeting siRNA- Human, Dharmacon). Accell- modified siRNAs are used without transfection reagents in serum free conditions. siRNAs were re-suspended in complete William E medium at a final concentration of 1 µM for the pool of EZH2 siRNA or non-targeting siRNA and the mixtures were added to the cultured HFs. 96 hours after transfection half of the HFs (5-12) from each group were collected for RNA isolation to evaluate transfection efficiency. The other half were embedded in cryomatrix and snap frozen for immunohistochemical analysis. Images were taken at day 0, 3 and 7 of the experiment using a stereoscopic microscope at x2 magnification equipped with a Nikon Coolpix digital camera for morphological evaluation. Images were collected using the Visicam image analysis software. The siRNA sequences are listed in Table 2.2.

Table 2.2 siRNA Target Sequences

siRNA	Target Sequence (5'-3')
ON-TARGETplus SMARTpool siRNA EZH2 Human	GAGGACGGCUUCCCAAUAA GCUGAAGCCUCAAUGUUUA UAACGGUGAUCACAGGAUA GCAAAUUCUCGGUGUCAAA
ON-TARGETplus Non-targeting pool	UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA
Accell Human EZH2 siRNA-SMARTpool	UUAUGAUGGUUAACGGUGA GCUGAAGCCUCAAUGUUUA CCUCUAACCAUGUUUACAA UGUCUGGAAUCAAAGGAUA
Accell Red Non-targeting siRNA	UGGUUUACAUGUCGACUAA
EED Trilencer-27 Human siRNA	GGACCUAUGAUAGCAAUACGAGCCA CGCUGUCAGUAUAGAAAGUGGUACA CCCUGAUACACCUACAAACACGCCA
Trilencer-27 Universal Scrambled Negative Control siRNA Duplex	CGUUAAUCG CGUAUAAUACGCGUAT

2.6 Treatment of cells and hair follicles with EZH2 and/or EZH1/2 inhibitors

NHEKs were treated with EZH2 and/or EZH1/2 inhibitors to analyse their effect on proliferation, differentiation and apoptosis. GSK126 (EMD Millipore Corp, USA) was utilised as a selective small molecule inhibitor of EZH2 (McCabe et al., 2012), UNC1999 (Tocris, UK) was utilised as a selective small molecule inhibitor of both EZH1 and EZH2 while UNC2400 (Tocris) was a negative control (Konze et al., 2013). Cells were seeded at an appropriate density in 6 or 12-well cell culture plates and treated with GSK126 at concentrations of 2 or 3μM, UNC1999 and UNC2400 at a concentration of 3μM. After 72 or 96 hours, cells were subsequently analysed for gene expression by qPCR and/or Western blot.

For HFs, microdissected human anagen HFs were treated with DZNep at concentrations of 5μM and 10μM. Images were taken of hair follicles on day 0 before treatment, day 3 and 6 after treatment. Images were captured using a Nikon Eclipse TS100 Light microscope (Nikon Instruments Inc., USA) and Vicicam camera (VWR, UK). Hair follicle length was measured using ImageJ software (NIH Image analysis website http://rsb.info.nih.gov/ij/). HFs were snap frozen and sectioned for use in Immunohistochemical analysis.

2.7 Plasmid Construction and Amplification

Human EZH2 cDNA (GenBank #BC010858) in pLenti-GIII-CMV-GFP-2A-Puro vector was purchased from Applied Biological Materials Inc (Richmond,

BC, Canada) and used as template to produce blunt-end PCR products using Phusion™ DNA polymerase (New England Biolabs, UK) and the following PCR primers:

F-5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGCCAGAC-TGGGAAG-3′

R -5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAGGGATTTCCAT-TTCTC-3′.

The resulting EZH2 cDNA was cloned in a pDONR™221 vector (Invitrogen), amplified by transformation in competent cells (Invitrogen) and sub cloned into destination vector pLKO.DEST.EGFP (Addgene plasmid #32684) using gateway™ cloning system (Invitrogen) as previously described (Radulovich et al., 2011). All constructs were verified by sequencing (University of Dundee DNA Sequencing and Services, Dundee, UK).

2.8 Lentiviral particles production and cell culture

For lentiviral particle production, HEK 293T cells were transfected with transfection complex made up of 2µg EZH2 expression vector DNA, lentiviral packaging mix (1.6µg psPAX2 [Addgene plasmid #12260], 500ng pMD2.G [Addgene plasmid #12259]) and 10µl PEI transfection reagent (Sigma-Aldrich, Dorset,UK) in 400µl Opti-MEM (Gibco). Two days after transfection, the virus-containing cell culture supernatant was collected, filtered using 0.45µm filter, concentrated by adding Peg-it™ virus precipitation solution (SBI, California, USA), incubated at 4°C overnight and centrifuged at 1500xg to pellet precipitated viral particles. The viral particles were aliquoted and

used for infection or stored at -80°C for later use. Transfection efficiency was evaluated 48 hours post transfection by the percentage of GFP positive cells using flow cytometry.

NHEKs stably expressing EZH2 or GFP (control) were generated by seeding NHEKs in 6-well plates and incubating at 37°C with 5% CO_2 until 60-70% confluent, then infected with EZH2 lentivirus or control lentivirus and polybrene (8µg/ml) and used for differentiation experiments.

2.9 Keratinocyte differentiation assay

Calcium has been identified as a trigger for keratinocyte differentiation (Pillai et al., 1988). NHEKs untreated, treated, transfected or infected were cultured in KBM2 medium containing 1.8mM $CaCl_2$ for 24, 48 and 72 hours depending on the nature of the experiment. Control NHEKs were always cultured under low Ca^{2+} conditions (0.06mM $CaCl_2$). Cells were subsequently analysed for gene expression by RT-qPCR and/or Western blot analyses.

2.10 *In vitro* model of differentiated and undifferentiated NHEKs

For the *in vitro* model for investigating the role of EZH2 in differentiated and undifferentiated human keratinocytes, juvenile keratinocytes (Promocell) were transfected with siRNA targeting EZH2 and treated with GSK126 in low Ca^{2+} medium (0.06mM). The medium was changedto a high Ca^{2+} medium (1.8mM) after 24hrs and cultured for another 48hrs. This was known as undifferentiated NHEKs (UD EZHi). A second group of juvenile keratinocytes

was cultured in high Ca^{2+} medium. After 24hrs cells were transfected with siRNA targeting EZH2 and treated with GSK126 andmaintained in high Ca^{2+} medium for another 48hrs. This was known as differentiated NHEKs (D EZHi). The control NHEKs were transfected with non-targeting siRNA under similar conditions (UD control and D control). Cells from all four groups were analysed 72hrs after the start of the experiment for gene expression by RT-qPCR and Western blot analyses.

2.11 Nuclear Extraction

Nuclear extracts were prepared from NHEKs induced to differentiate in the presence of high Ca^{2+} by using EpiQuik™ Nuclear Extraction Kit (Epigentek Group Inc., Brooklyn, NY, USA) following the manufacturer's instruction. Briefly, nuclear pellets were prepared from adherent cells and suspended in extraction buffer containing DTT and protease inhibitor cocktail. Suspension was centrifuged for 10 minutes at 14,000 rpm at 4°C. Supernatant was transferred into Eppendorf tubes. Nuclear extracts were quantified using the Qubit protein assay kit and Qubit 2.0 fluorometer (ThermoFisher Scientific) following manufacturer’s instructions. Quantified nuclear extracts were either stored at -80°C for future use or used immediately for HMTase activity assay.

2.12 HMT catalytic activity detection

HMT activity was assayed by using EpiQuik™ Histone Methyltransferase Activity/Inhibition Assay Kit (H3-K27) (Epigentek, USA) following the

manufacturer's instruction. HMT activity was calculated by using the following formula:

$$HMT\ Activity(ng/h/mg) = \frac{OD(sample - blank)}{Protein\ amount(\mu g) \times hour \times slope} \times 1000$$

2.13 Immunocytochemistry

To assess cell proliferation, 5-bromo-2'-deoxyuridine (BrdU) assay that is based on incorporation of BrdU into cellular DNA during cell proliferation and detection of phosphorylated histone 3 on serine 28 in mitotic cells was utilised. To assess apoptosis, activated caspase 3 detection in apoptotic cells was utilised. Briefly, NHEKs were seeded on 15X15mm sterile coverslips (Menzel Glaser) in 12 well plates and cultured in 1ml complete KBM2 medium (PromoCell GmbH). Coverslips were coated with collagen prior to apply growing cells. Cells were seeded at an appropriate density and grown until 60 - 70% confluence was achieved. Cells were either transfected with control siRNA and EZH2 siRNA at a concentration of 50nM or treated with GSK126, UNC1999 and UNC2400 at a concentration of 3µM. Cells were allowed to proliferate for 48 - 72 hours prior to fixation and permeabilization. Coverslips were incubated with antibodies directed against proliferation and apoptosis markers, FITC-Anti-Bromodeoxyuridine[BrdU] (Abcam), H3S28p (Cell signalling) and activated Caspase 3[Caspase 3a] (Abcam). Cell nuclei were stained using DAPI [4', 6-Diamidino-2-phenylindole] (Vector Laboratories, UK) or Propidium iodide [PI] (Sigma).

Cells were visualized using a Nikon ECLIPSE 50i fluorescent microscope (Nikon Instruments Inc., USA) and analysed using Image Pro Express (Media Cybernetics). Images of coverslip mounted cells were taken at X40 magnification (n=5). The percentage of positive proliferating or mitotic cells (BrdU+ or H3S28p+) and positive apoptotic cells (Caspase 3a+) were calculated from the total number of DAPI+ or PI+ cells.

2.14 Protein concentration determination

Protein concentrations in cell lysates were determined by Bradford assay (ThermoFisher Scientific, Cramlington, UK) using manufacturer's protocol. Titrated concentrations of bovine serum albumin (BSA) were used as standard. 5µl of standards and lysates were aliquoted into a flat bottomed 96-well plate (Corning). 200µl Coomassie protein reagent was added to the wells. The intensity of colour change from violet red to blue indicated the amount of protein present. Absorbance was measured at 560nm using Magellan 6, 200 series (Tecan).

2.15 Western Blot Analysis

Keratinocytes cultured in 6-well plates were lysed with CelLytic M lysis buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Roche Applied Bioscience, Rotkreuz, Switzerland) to prevent protein degradation. Protein concentration was determined by Bradford assay (ThermoFisher Scientific). Total protein was incubated in 4X Loading buffer (200mM Tris Base, pH6.8, 40% v/v glycerol, 8% w/v SDS, 0.04% w/v bromophenol blue and 20% v/v

beta-mercaptoethanol) for 5min at 95°C. 20-30µg of total protein was separated according to their molecular weight using 10-12 % SDS-PAGE. Proteins were blotted onto Nitrocellulose membrane (GELifesciences) using the wet transfer method. Membrane was blocked in 3% w/v dried semi-skimmed milk in 1XTBST (10mM Tris Base pH7.2, 0.15M NaCl and 0.1% v/v Tween-20) for 1 hour on a shaker at 4°C. Membrane was probed with primary antibodies (listed in table 2.3) in 1XTBST containing 3% w/v dried semi-skimmed milk overnight at 4^{o}C with gentle shaking. HRP-conjugated secondary antibodies (listed in table 2.4) were used at 1:2000 in 1xTBST for 1 hour at 4^{o}C with gentle shaking. Membranes were incubated in Super Signal West Femto substrate (Thermo Scientific) for 5 minutes at room temperature and protein bands were detected using Chemidoc imaging system (Bio-Rad). Densitometric analysis was performed on western blot images using ImageJ software (NIH Image analysis website http://rsb.info.nih.gov/ij/). Quantification provided as the ratio to actin abundance as a normalization control.

For a repeat Western blot for loading control, membranes were stripped at room temperature for 30 minutes using stripping buffer (25mM Glycine, 1% SDS, 0.02% Sodium Azide and 0.1% β-Mercaptoethanol) and blocked with 3% w/v dried semi-skimmed milk in 1XTBST for 30 minutes prior to probing with primary and secondary antibodies (listed in tables 2.2 and 2.3).

2.16 Immunohistochemistry

Cryosections (10µm-thick) were dried at room temperature for 10 minutes, encircled with ImmEdge hydrophobic barrier pen (Vector Laboratories, UK) and fixed for 10 minutes in Acetone or Acetone/Acetic acid (1:1 v/v) at -20°C or 4% v/v Paraformaldehyde (PFA) at room temperature depending on the primary antibody used. Slides were washed 3 times for 5 minutes at room temperature in 1X PBS. Slides were incubated at room temperature for 35 minutes with either 2.5% w/v Bovine Serum Albumin (BSA) or 10% v/v Donkey serum. Primary antibodies (listed in Table 2.3) were diluted in blocking buffer consisting of 5% w/v Bovine Serum Albumin (BSA), 1% v/v Triton-X100 and 2% w/v Saponin in 1X PBS. 100µl of each primary antibody solution was added onto slides with tissue sections and incubated overnight at 4°C. Slides were washed 3 times for 5 minutes at room temperature in 1X PBS with 0.05% v/v Triton-X100. Relevant secondary antibodies (listed in Table 2.4) diluted in blocking buffer were used for detection of primary antibodies. Slides were incubated for 1 hour at 37°C, washed 3 times for 5 minutes in 1X PBS with 0.05% v/v Triton-X100 and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, UK) or Fluoroshield mounting medium (Abcam). Immunofluorescence was detected using a Nikon ECLIPSE50i fluorescence microscope (Nikon Instruments Inc., USA) and image analysis software, Image Pro Express (Media Cybernetics).

Table 2.3 Primary Antibodies and associate dilutions

ANTIGEN	APPLICA-TION	HOST	DILUTION USED	SUPPLIER
EZH2 #Ab3748	IHC ICC WB	Rabbit	1:250 1:1000 1:1000	Abcam
EZH1 #SC-20347	WB	Goat	1:1000	Santa Cruz
PCNA #2586	IHC	Mouse	1:100	Cell Signalling
ACTIVE Caspase 3 #Ab13847	IHC ICC	Rabbit	1:100/1:500	Abcam
FITC-α BrdU #347583-7583	ICC	Rabbit	1:2000	Beckton Dickinson, USA
Cytokeratin 1 (K1) #Ab93652	WB	Rabbit	1:1000	Abcam
Cytokeratin 10 (K10) #M7002 #Ab76318	IHC WB	Mouse Rabbit	1:100 1:2000	Dako Abcam
Cytokeratin 14	IHC	Mouse	1:100	Abcam

(K14) #Ab7800				
Cytokeratin 15 (K15) #SC47697	IHC	Mouse	1:100	Santa Cruz
Cytokeratin 19 (K19) #V1074	IHC	Mouse	1:100	Biomeda Corp
Loricrin #PRB-145P	WB	Rabbit	1:1000	Biolegend
H3K27me3 #9733s	WB	Rabbit	1:1000	Cell Signalling
CD200 #AF2724	IHC	Goat	1:100	R & D system
Actin #Ab3280	WB	Mouse	1:2000	Abcam
GAPDH #Ab8245	WB	Mouse	1:2000	Abcam
α6-intergrin- PE #555736	FACS	Rat	1:25	BD Pharmigen
Sca1-FITC #11-5981-85	FACS	Rat	1:50	eBioscience

Table 2.4 Secondary Antibodies and associate dilutions

ANTIBOD-IES	APPLICAT-ION	HOST/ SPECIFICITY	DILUTION USED	SUPPLIER
Alexa 488 #A11029	IHC	Goat α Mouse	1:200	Invitrogen
Alexa 555 #A31572	IHC ICC	Donkey α Rabbit	1:200 1:1000	Invitrogen
HRP-Conjugated #7074s	WB	Horse α Rabbit	1:2000	Cell Signalling
HRP-Conjugated #7076s	WB	Horse α Mouse	1:2000	Cell Signalling
HRP-Conjugated #R-21459	WB	Rabbit α Goat	1:2000	Life Technology

2.17 Total RNA Isolation

Total RNA was isolated from untreated, treated and transfected primary human epidermal keratinocytes or human hair follicles using the Direct-zol RNA MiniPrep kit (Zymo Research Corp, USA) following manufacturer's protocol. Briefly, adherent cells were lysed directly in cell culture plates by adding 500 -1000µl of TRI Reagent (Zymo Research Corp), scraping cells

using a cell scraper and transferred into RNase-free tubes. Hair follicles were put in TRI Reagent and mechanically homogenized with a pestle. The homogenate from either adherent cells or human hair follicles was incubated at room temperature (RT) for 5 minutes to allow the complete dissociation of nucleoprotein complexes. Ethanol was added directly to the homogenate (1:1), vortexed to mix and loaded into Zymo-Spin IIC Column in a collection tube. After centrifugation at 14,000xg for 30 seconds, the column was transferred to a new collection tube. 400µl of RNA Wash Buffer was added to the column and centrifuged at 14,000xg for 30 seconds to wash the column. 80µl of DNase I Reaction Mix was added directly to the Column matrix and incubated at RT for 15 minutes. 400µl of RNA PreWash Buffer was added to the column and centrifuged at 14,000xg for 30 seconds to wash the column. This wash was done twice. 700µl of RNA Wash Buffer was added to the column and centrifuged at 14,000xg for 2 minutes and the column was transferred to an RNase-free tube. RNA was eluted with 25 - 50µl of nuclease-free water. RNA was quantified immediately using a Nanodrop (ThermoFisher Scientific). Quantified RNA was used immediately for complementary DNA synthesis or stored at -80°C for use later.

2.18 Complementary DNA (cDNA) Synthesis

Following RNA quantification, complementary DNA (cDNA) was generated for each total RNA isolated using qPCRBIO cDNA Synthesis kit (PCRBiosystems Limited, UK) following manufacturer's protocol. Each 20ml reverse transcription reaction mix contained 4µl of 5X cDNA synthesis mix,

1µl of 20X reverse transcriptase (RTase), total RNA (between 100ng and 400ng) and nuclease-free water up to 20µl final volume. Tubes were briefly centrifuged and loaded into the Thermal Cycler (Eppendorf, UK) and the reverse transcription protocol started as follows: incubation at 42°C for 30 minutes followed by incubation at 85°C for 10 minutes to denature the enzyme, reverse transcriptase. Synthesized cDNA samples were stored at -20°C until analysed.

2.19 Real-Time Quantitative PCR (RT-qPCR) Analysis

RT-qPCR primers were designed using PRIMER3web design software and ordered from Sigma. All primers were optimised using human universal reference cDNA (Clontech) at six different temperatures – 60°C, 61°C, 62°C, 63°C, 64°C and 65°C. Melting curves were compared to establish optimal annealing temperatures for each primer.

Each 20µl real-time PCR reaction mix contained 2µl of cDNA, 10µl of 2X qPCRBIO SyGreen Hi-ROX mix (PCRBiosystems, UK), 4.8µl of nuclease-free water (Gibco) and 3.2µl of forward and reverse primers (Listed in Table 2.5).

All real-time PCR amplifications were performed using StepOnePlus Real-Time PCR System (Applied Biosystems, UK) with duplicate or triplicate samples for each gene of interest. GAPDH or YWHAZ was used as housekeeping gene for normalization. Data was exported to Excel and analysed according to the ΔCt method using a reference gene.

Firstly, Ct values of each sample replicates were normalised using GAPDH or YWHAZ as a reference gene:

ΔCt [normalised samples] = (Ct [GAPDH or YWHAZ] – Ct [Target])

Next, the linear conversion of the normalised samples ΔCt was calculated ($2^{(-\Delta Ct)}$). The average of the $2^{(-\Delta Ct)}$ values, SD, SEM were then calculated.

Table 2.5 Real-Time quantitative PCR primers

SEQUENCE DEFINITION	SEQUENCE (5' – 3')	ANNEALING T°C
EZH2	F-CAGAAGTTGAACGTGGGCAA R-GTCCTCCACCAACCTCTTCA	60°C
EZH1	F-GAGTTGGTCGATGCCCTGAAT R-AGCATGTCGCTTTCTCTTTCTT	62°C
Keratin 1 (KRT1)	F-ACTTGACAACTTGCAGCAGG R-CAATGATGCTGTCCAGGTCG	60°C
Keratin 10 (KRT10)	F-TTCGGGCTCTGGAAGAATCAAAC R-GCAGGATGTTGGCATTATCAGTTG	61°C
Keratin 14 (KRT14)	F-GCCTGTCTGTCTCATCCTC R-CTGAAGCCACCGCCATAG	61°C
Keratin 15 (KRT15)	F-AAAGAAGGGCCTCCAACACA R-TCTCGGCCAGTGAGTTCTCC	62°C
Keratin 19 (KRT 19)	F-GTCGATCTGCAGGACAATCC R-CCGCGACTACAGCCACTACT	62°C

Loricrin	F-ACGGAGGCGAAGGAGTT R-CCAGGTAGGTTAAGACATGAAGG	64°C
RPTN	F-TGAGTCACAAATCTACCAGTGGC R-ACTGTCCACAATAAGAGCCTGAT	60°C
FOXN1	F-CCTTCGAGGAGATCCCAGTG R-TAGGGGAGCCCGTTACACC	62°C
CXCL10	F-GTGGCATTCAAGGAGTACCTC R-TGATGGCCTTCGATTCTGGATT	60°C
SPRR3	F-GGCTGAACACCTCGACCTT R-GCTTCAAAGGATGCTGGACACAC	61°C
Keratin 13 (KRT13)	F-CCCCAGGCATTGACCTGAC R-GTGTTGGTAGACACCTCCTTG	61°C
TINCR	F-TGTGGCCCAAACTCAGGGATACAT R-AGATGACAGTGGCTGGAGTTGTCA	60°C
FLG	F-AAAGAGCTGAAGGAACTTCTGG R-AACCATATCTGGGTCATCTGG	60°C
CCND1	F-CAATGACCCCGCACGATTTC R-CATGGAGGGCGGATTGGAA	61°C
TAB2	F-CTCCTGGTGGTACAACTCGAC R-TGATTTGGCTGTTGAGATGAGG	60°C
TREM2	F-GAGAAATCCCCTTCCCACCC R-GTGTCTCTCAGCCCTGGCA	60°C
$p16^{INK4A}$	F-ATGGAGCCTTCGGCTGACT R-GTAACTATTCGGTGCGTTGGG	62°C
$p19^{INK4D}$	F-GATGTCAACGTGCCTGATGG R-CCCTGCGATGGAGATCAGAT	62°C
$p14^{ARF}$	F-GGGTTTTCGTGGTTCACATCC	61°C

	R-CTAGACGCTGGCTCCTCAGTA	
GAPDH	F- TTGAGGTCAATGAAGGGGTC R- GAAGGTGAAGGTCGGAGTCA	61°C
YWHAZ	F-TTCTTGATCCCCAATGCTTC R-AGTTAAGGGCCAGACCCAGT	61°C
PCNA	F-AGCCGAAACCAGCTAGACTT R-GATGAGGTCCTTGAGTGCCT	60°C
CD200	F-GAACCATGGGGTGGTGATCC R-GGCTGTACATAGACGGTGAGG	63°C
CD34	F-CAGGAGAAAGGCTGGGCGAA R-ATTCGGTATCAGCCACCACG	63°C

2.20 Fluorescent-Activated Cell Sorting Analysis (FACS)

FACS analysis was performed as previously described (Jensen et al., 2013). Briefly, human skin strips were collected and subjected to 5mg/ml Dispase (Gibco) digestion overnight at 4°C followed by 0.25% trypsin for 14 minutes at 37°C with moderate shaking. Cell suspension was passed through a cell strainer, and cells were then stained with α6-integrin-PE (1:25) and Sca1-FITC (1:50). Basal cells were sorted as Sca1 (+), a6-integrin (+) and suprabasal cells sorted as Sca1 (-), a6-integrin (-). The dead cell population was excluded by DAPI staining. Sorting was performed on Moflo XDP cell sorter (Beckman Coulter, Buckinghamshire, UK) at 35 psi with a 100-mm

nozzle and sorted cells were collected directly into Trizol™ LS (Life Technology) for total RNA isolation.

2.21 Microarray and Bioinformatics Analysis

Total RNA was extracted from the adult keratinocytes and juvenile keratinocytes 24 and 48 hours after EZH2 siRNA transfection as described above. Quality of total RNA was tested by capillary electrophoresis on an Agilent 2100 bioanalyzer (Agilent). Samples were sent to EMBL GeneCore for Affymetrix Microarray analysis (EMBL GeneCore, Germany). The Raw fluorescence intensity values were normalized applying quantile normalization and RMA background correction. ANOVA was performed to identify differential expressed genes using a commercial software package SAS JMP10 Genomics, version 6, from SAS (SAS Institute, Cary, NC, USA). A false positive rate of a=0.05 with FDR correction was taken as the level of significance.

2.22 Statistical Analysis

Data were analysed for statistical significance using analysis of variance (ANOVA) and compared columns using Bonferroni when the graph involves multiple comparison or *t*-test when data are normally distributed and only two sets of data are compared or Mann Whitney U test when data are not normally distributed and only two sets of data are compared on Graphpad prism statistical analysis software (GraphPad Prism 6, GraphPad Software, San Diego, CA).‘n’ represents the number of independent experiments done.

All data are expressed as either Means ± SD or Means ± SEM. Values of $p < 0.05$ were considered significant. *p< 0.05, **p< 0.01, ***p< 0.001, ****p<0.0001.

Chapter 3: Polycomb Repressive Complex 2 is required for effective epidermal differentiation in human epidermis

3.1 Introduction

The human epidermis acts as a barrier protecting the body from the external environment, against fluid loss, toxic chemical exposure, infectious agents, mechanical damage and ultraviolet radiation (Costin and Hearing, 2007; Tobin, 2006). The formation of a stratified epidermis in humans is initiated during embryonic development and continues throughout the life time of individuals. This continuous stratification of the human epidermis involves a cascade of cellular events that includes keratinocyte proliferation, terminal differentiation, migration, autophagy and desquamation (Akinduro et al., 2016; Watt et al., 1988; Zhang et al., 2015). Basal mitotically active keratinocytes expressing K5 and K14 exit the cell cycle and commit to a terminal differentiation programme that gives rise initially to the spinous keratinocytes expressing K1 and K10 and then granular keratinocytes expressing Loricrin and Filaggrin, which eventually undergo cornification to form corneocytes which desquamate from the topmost layer of the epidermis, the stratum corneum (Bowden et al., 1987; Watt et al., 1988).

Polycomb repressive complex 2 (PRC2) is a chromatin repressor that plays significant role in the regulation of tissue development, stem cell fate control

and in a variety of diseases such as cancer (Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010; Surface et al., 2010). Mammalian PRC2 consists of core subunits embryonic ectoderm development (EED), suppressor of zeste 12 homolog (SUZ12) and enhancer of zeste homolog 1/2 (EZH1/2). EZH1/2 is the methyltransferase component of PRC2 responsible for catalysing the trimethylation of lysine 27 on histone H3 (H3K27me3) repressive histone mark. EZH2 carboxyl-terminal Su(var) 3,9, enhancer of zeste, trithorax (SET) domain is the catalytic domain required for methyltransferase activity (Cao et al., 2002; Kuzmichev et al., 2002). EED and SUZ12 are required for proper HMTase activity of EZH2 (Cao and Zhang, 2004; Han et al., 2007; Montgomery et al., 2005; Pasini et al., 2004).

Loss of function studies in mouse have revealed an essential role for PRC2 as a regulator of differentiation in mammalian skin development (Bardot et al., 2013; Dauber et al., 2016; Ezhkova et al., 2011; Ezhkova et al., 2009; Perdigoto et al., 2016). Based on these findings, it has been hypothesized that PRC2 could also be an essential regulator of epidermal differentiation in humans. In this project, the role of PRC2 in human skin homeostasis is investigated by examining the roles of EZH1/2 and EED in keratinocyte proliferation, differentiation, apoptosis and aging in human epidermis.

3.2 Results

3.2.1. EZH2 expression is upregulated in differentiating suprabasal keratinocytes in human epidermis

To determine a potential role for EZH2 in human skin homeostasis, its expression pattern was evaluated in adult human skin tissues by utilising a wide range of methods, including Immunohistochemistry, immunocytochemistry, FACS and RT-qPCR analysis.

Immunofluorescence analysis revealed that EZH2 is expressed in both basal and suprabasal keratinocytes of adult human epidermis with little or no expression in the dermis. Interestingly, EZH2 was more prominently expressed in K10+ suprabasal keratinocytes compared to K14+ basal keratinocytes (Fig. 3.2.1A-B).

The nuclear expression of EZH2 was also confirmed in NHEKs cultured on coverslips in serum-free medium *in vitro* (Fig.3.2.1C). Growing NHEKs in serum-free medium prolong their proliferative state (Pillai et al., 1988).

To further confirm EZH2 expression pattern in human epidermis, keratinocytes isolated from fresh human skin tissue was sorted by FACS using α6 integrin (CD49f) and Sca1 antibodies as described previously (Jensen et al., 2013). Basal keratinocytes were $\alpha6^+$, $Sca1^+$ while suprabasal keratinocytes were $\alpha6^-$ and $Sca1^-$. Expression of EZH2 and markers of the basal and suprabasal layers in FACS sorted basal and suprabasal keratinocytes were analysed by RT-qPCR analysis. EZH2 expression was

significantly upregulated in suprabasal keratinocytes compared to basal keratinocytes. Furthermore, K14, a marker of the basal layer was downregulated while K1 and Loricrin, markers of the suprabasal layer were upregulated in suprabasal keratinocytes compared to the basal keratinocytes (Fig. 3.2.1D), confirming the purity of the two populations of keratinocytes.

Taken together, these results suggest that EZH2 expression is upregulated in differentiating suprabasal keratinocytes compared to their progenitors in the basal epidermal layer.

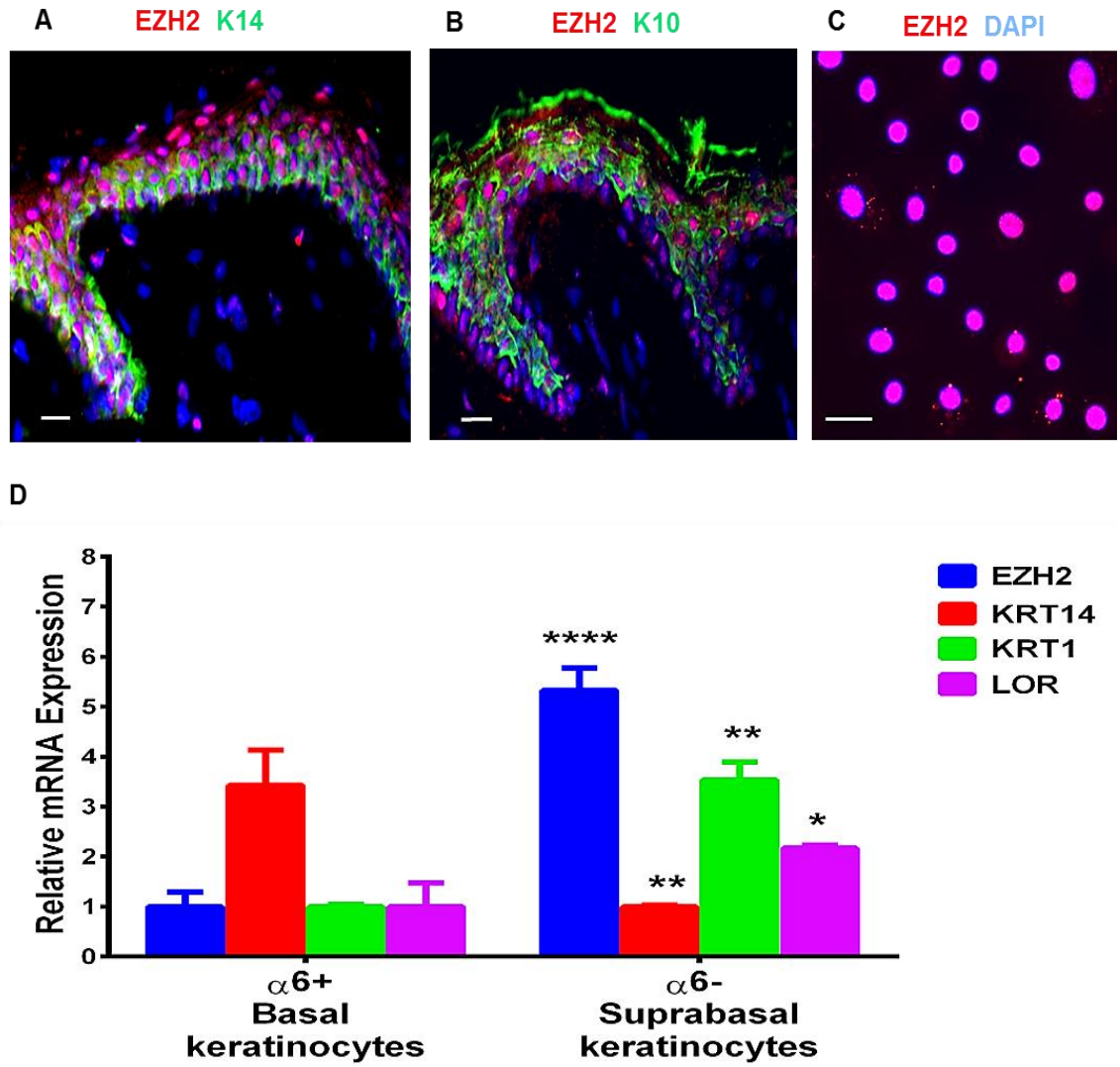


Figure 3.2.1. EZH2 expression is upregulated in differentiating suprabasal keratinocytes in human epidermis. *(A-B)* Double Immunofluorescence for EZH2 and K14 (A) and K10 (B) showing EZH2 expression in K14+ basal keratinocytes but increased expression in K10+ suprabasal keratinocytes. (C) Immunostaining showing EZH2 nuclear expression in NHEKs grown on coverslips in serum-free medium. (D) RT-qPCR in FACS-purified human epidermal keratinocytes showing an upregulation of *EZH2*, *KRT1*, *LOR* and downregulation of *KRT14* in α6⁻ suprabasal keratinocytes compared to α6+ basal keratinocytes (Mean±SD; n=3; ****P<0.0001, **P<0.01, *P<0.05). Scale bars: (A-B) 50μm, (C) 100μm

3.2.2. EZH2 expression and enzymatic activity increases in differentiating keratinocytes *in vitro*

To investigate a potential role for EZH2 in the control of keratinocyte differentiation, NHEKs were cultured in low (0.06mM) and high (1.8mM) Ca^{2+} medium for 24 hours, 48 hours and 72 hours. In culture, NHEKs need between 0.03 – 0.15mM of Ca^{2+} to proliferate while elevated levels of Ca^{2+} above 1mM induce their differentiation (Pillai et al., 1988). Effects of elevated Ca^{2+} on gene expression were analysed by RT-qPCR and Western blot.

A significant up-regulation of EZH2 expression was observed in keratinocytes cultured in high Ca^{2+} medium compared to the less differentiated keratinocytes grown in low Ca^{2+} medium after 72 hours (Fig. 3.2.2A). Ca^{2+}-induced differentiation was confirmed by the increased expression of K10 protein level in keratinocytes grown in high Ca^{2+} medium (Fig. 3.2.2B-C). Expression of EZH2 protein level as well as H3K27me3 level was increased in keratinocytes grown in high Ca^{2+} medium compared to keratinocytes grown in low Ca^{2+} medium (Fig. 3.2.2B-C). Furthermore, a concurrent upregulation of EZH1 protein level, another PRC2 component, was observed in keratinocytes grown in high Ca^{2+} medium compared to keratinocytes grown in low Ca^{2+} medium (Fig. 3.2.2B-C).

In addition, the effect of Ca^{2+}-induced keratinocyte differentiation on EZH2 methyltransferase activity (HMTase Activity) was also evaluated utilising histone methyltransferase activity assay.

A 3-fold increase in HMTase activity was observed in differentiated keratinocytes grown under the high Ca^{2+} condition for 72 hours compared to

the less differentiated keratinocytes grown in low Ca^{2+} condition (Fig. 3.2.2D), suggesting that an increase in Ca^{2+} concentration might correlate with increased EZH2 enzymatic activity.

Taken together, these results suggest that not only is EZH2 expression upregulated in differentiating keratinocytes but also EZH2 enzymatic activity.

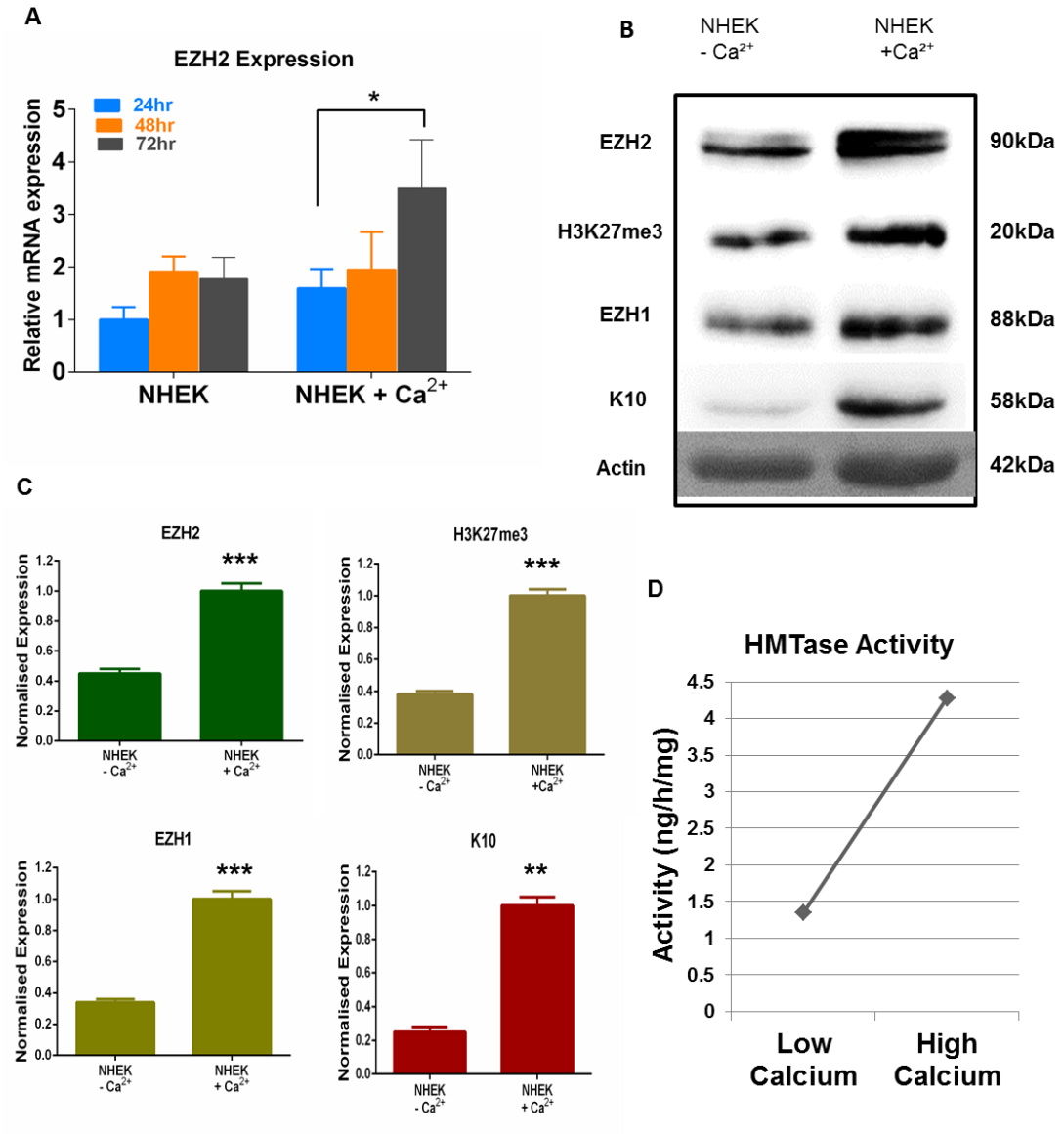


Figure 3.2.2. EZH2 expression and enzymatic activity increases in differentiating keratinocytes *in vitro*. (A) RT-qPCR showing upregulation of EZH2 in differentiating keratinocytes during Ca^{2+} -induced differentiation (Mean±SD; n=3; *P<0.05). (B-C) Western blot and densitometric analysis showing increased EZH2, H3K27me3, EZH1 and K10 protein levels in differentiating keratinocytes during Ca^{2+} -induced differentiation. Actin was used as a loading control (Mean±SD; n=3, **P<0.01, ***P<0.001). (D) HMTase activity assay showing increased HMTase activity in differentiating keratinocytes during Ca^{2+} -induced differentiation. NHEK - Normal human epidermal keratinocytes. HMTase – Histone Methyltransferase

3.2.3 EZH2 controls epidermal differentiation by preventing the premature expression of differentiation genes in basal keratinocytes

To assess whether EZH2 plays a role in epidermal differentiation, small interfering RNA (siRNA) targeting EZH2 was utilised to knockdown EZH2 expression in NHEKs. Gene expression was analysed by RT-qPCR.

RT-qPCR analysis revealed a marked increase in the expression of differentiation genes, *KRT1* and *LOR* in EZH2-depleted keratinocytes cultured in low Ca^{2+} medium compared to keratinocytes transfected with a non-targeting siRNA (Control siRNA) grown under same conditions for 24 hours and 72 hours after transfection (Fig.3.2.3A), suggesting that EZH2 is involved in regulating the expression of these differentiation genes in undifferentiated keratinocytes.

To further assess the functional role of EZH2 in terminal differentiation, EZH2 was silenced in undifferentiated keratinocytes utilising either siRNA or a small molecule EZH2 inhibitor GSK126 approaches. Transfected or treated Keratinocytes were either induced to differentiation by high Ca^{2+} conditions or maintained in low Ca^{2+} conditions 24hrs post transfection or treatment. Effect of EZH2 depletion on gene expression was analysed by RT-qPCR and Western blot 48 hours post transfection or treatment.

RT-qPCR analysis revealed a significant upregulation of KRT*1* and *LOR* expression in EZH2 depleted keratinocytes grown in low Ca^{2+} medium compared to the untreated or non-targeting siRNA transfected keratinocytes grown under same conditions (Fig.3.2.3B-C). In addition, a significant further

increase in the expression of *KRT1* and *LOR* was observed in EZH2 depleted keratinocytes upon Ca^{2+} - induced differentiation compared to the corresponding control (Fig. 3.2.3B-C).

Transfection efficiency and Ca^{2+}-induced differentiation was confirmed by reduced expression of EZH2 protein level and increased expression of K1 and LOR protein levels in EZH2-depleted keratinocytes compared to the corresponding control upon Ca^{2+}-induced differentiation (Fig. 3.2.3D-E).

Taken together, these results suggest that EZH2 controls epidermal differentiation by preventing the premature expression of differentiation genes in basal keratinocytes.

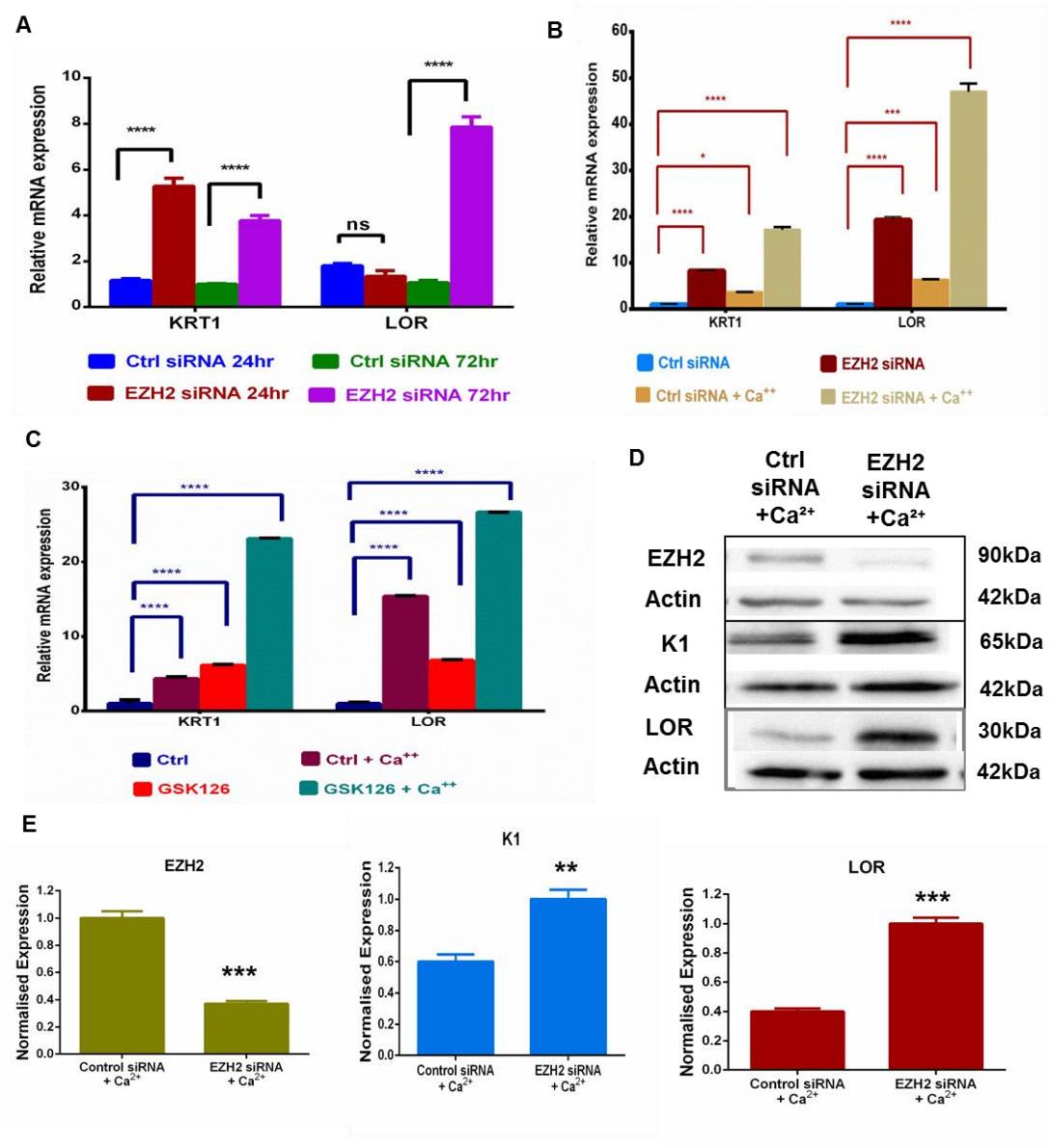


Figure 3.2.3. EZH2 controls epidermal differentiation by preventing the premature expression of differentiation genes in basal keratinocytes. (A) RT-qPCR showing significant upregulation of differentiation markers *KRT1* and *LOR* after EZH2 knockdown with siRNA in NHEKs (B-C) RT-qPCR showing marked upregulation of differentiation markers *KRT1* and *LOR* during Ca^{2+} -induced differentiation after EZH2 silencing with siRNA and a small molecule, GSK126 in NHEKs (Mean±SD; n=3, ns-not significant, ****P<0.0001, ***P<0.001, *P<0.05) (D-E) Western blot and densitometric analysis confirming knockdown of EZH2 and showing upregulation of K1 and LOR protein levels during Ca^{2+} -induced

differentiation after EZH2 knockdown with siRNA in NHEKs. Actin was used as a loading control (Mean±SD; n=3, **P<0.01, ***P<0.001). NHEKs- Normal human epidermal keratinocytes

3.2.4 Overexpression of EZH2 does not inhibit keratinocytes differentiation *in vitro*

To further investigate its functional role in terminal differentiation, EZH2 was overexpressed in undifferentiated NHEKs utilising a lentiviral vector either expressing EZH or expressing GFP as a control.

Infection efficiency was assessed by RT-qPCR, which revealed a 13-fold increase in EZH2 expression in EZH2-expressing keratinocytes compared to the GFP-expressing keratinocytes (Fig. 3.2.4A). However, EZH2 overexpression did not lead to an increase in the expression of *KRT1* and the lncRNA *TINCR,* while it resulted in a marked decrease in LOR expression compared to the corresponding control (Fig.3.2.4B), confirming earlier findings shown above that EZH2 prevents the premature expression of these genes.

To investigate the effect of EZH2 overexpression on keratinocyte differentiation, NHEKs infected with EZH2 lentivirus were induced to differentiate by high Ca^{2+} condition 24 hours post infection. Infection efficiency and gene expression was assessed after 72 hours by RT-qPCR.

RT-qPCR analysis revealed a 3-fold increase in *EZH2* expression in EZH2-expressing Keratinocytes grown in high Ca^{2+} medium compared to the corresponding control (Fig. 3.2.4C). In addition, a significant upregulation in the expression of *KRT1*, *LOR* and the lncRNA *TINCR* was observed in EZH2-expressing keratinocytes upon Ca^{2+}-induced differentiation compared to the corresponding control (Fig. 3.2.4D), suggesting that overexpressing EZH2 does not block Ca^{2+}-induced differentiation.

Taken together, these results suggest that overexpression of EZH2 in keratinocytes prevents the premature expression of differentiation associated genes but does not inhibit their ability to undergo Ca^{2+}-induced differentiation.

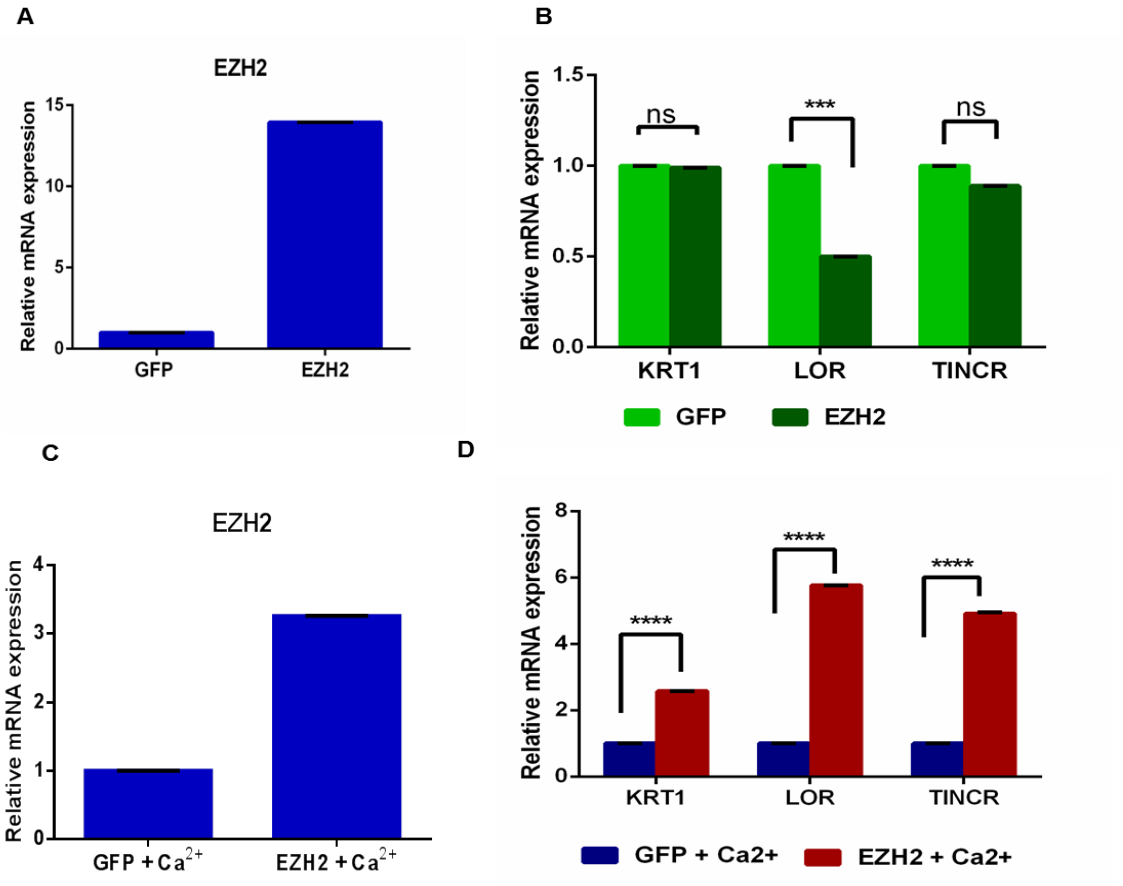


Figure 3.2.4. Overexpression of EZH2 does not inhibit keratinocytes differentiation *in vitro*. (A) RT-qPCR showing a 13-fold increase in EZH2 expression in EZH2-expressing keratinocytes compared to GFP-expressing keratinocytes (control) (B) RT-qPCR showing no increase in expression of *KRT1*, *LOR* and the lncRNA TINCR in EZH2-expressing keratinocytes compared to GFP-expressing keratinocytes (Mean±SEM, n = 3, P***<0.001) (C) RT-qPCR showing a 3-fold increase in EZH2 expression in EZH2-expressing keratinocytes compared to GFP-expressing keratinocytes (control) after Ca^{2+} -induced differentiation (D) RT-qPCR showing upregulation of *KRT1*, *LOR* and the lncRNA TINCR in EZH2-expressing keratinocytes compared to GFP-expressing keratinocytes upon Ca^{2+} -induced differentiation (Mean±SEM, n=3, P****<0.0001). EZH2 – EZH2 expressing Keratinocytes. GFP- GFP expressing keratinocytes (control).

3.2.5 EZH2 depletion mediates complex changes in gene expression program in primary keratinocytes

To further explore mechanisms underlying the effects of EZH2 on keratinocyte differentiation, microarray analysis of the global gene expression in keratinocytes after EZH2 silencing was performed. Total RNA from EZH2 siRNA transfected either juvenile or adult human keratinocytes was collected at 24 and 48 hours post transfection. Microarray profiling was followed by RT-qPCR validation.

Gene Ontology (GO) analysis revealed the alteration of pathways involved in keratinocytes differentiation, epidermis development, and keratinocytes proliferation (Fig. 3.2.4A). In addition, different classes of non-coding RNAs such as snoRNAs and rRNAs were differentially expressed in EZH2-depleted NHEKs (Fig. 3.2.4B). Interestingly, one of the upregulated ncRNA was the lncRNA TINCR (Tissue differentiation-Inducing Non-protein Coding RNA), which mediates keratinocyte differentiation (Kretz, 2013).

Differentially expressed genes selected for microarray validation by RT-qPCR included lncRNA *Tissue differentiation-Inducing non-protein coding RNA* (*TINCR), Filaggrin (FLG), Small Proline Rich Protein 3 (SPRR3), Keratin13 (KRT13), Repetin (RPTN), Triggering receptor expressed on myeloid cells 2* (*TREM2), C-X-C Motif Chemokine Ligand 10 (CXCL10)*, *Forkhead box protein N1(FOXN1), TGF-beta activated kinase 1/MAP3K7 binding protein 2 (TAB2)* and *Cyclin D1 (CCND1)*.

Microarray validation by RT-qPCR analysis confirmed that the expression of *TINCR, FLG, SPRR3, KRT13, RPTN, TREM2, CXCL10* and *FOXN1* was up-

regulated in EZH2 depleted NHEKs while the expression of TAB*2* and *CCND1* was downregulated (Fig. 3.2.4C-E). The changes in the expression of these genes in EZH2-depleted NHEKs compared to the control reflect their increased differentiated status.

Taken together, these results suggest that EZH2 mediates complex changes in gene expression program in primary human keratinocytes.

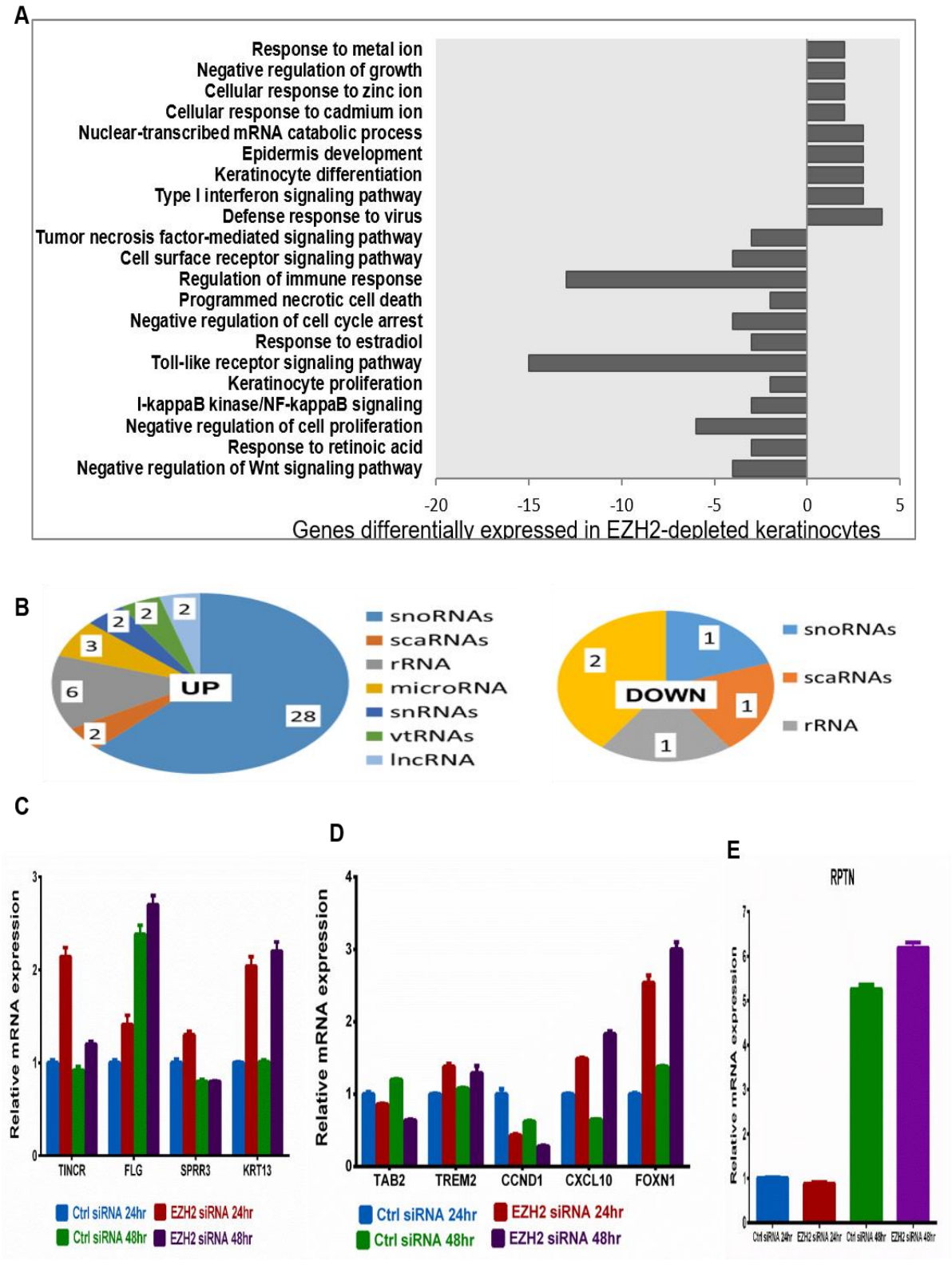


Figure 3.2.5. EZH2 depletion mediates complex changes in gene expression program in primary keratinocytes. (A) Gene Ontology (GO) terms enriched in EZH2-depleted NHEKs (DAVID 6.8

bionformatic analysis of Affymetrix microarray data*).* (B) Annotation of different classes of ncRNAs up-regulated and down-regulated in EZH2-depleted NHEKs (C-E) RT-qPCR analysis of selected genes in EZH2-depleted NHEKs showing changes in gene expression validating microarray data (Mean±SEM, n=3). NHEKs – Normal Human Epidermal Keratinocytes

3.2.6 EZH2 prevents the premature expression of terminal differentiation associated genes in undifferentiated basal keratinocytes and modulates the dynamics of differentiation in differentiated suprabasal keratinocytes

As shown in data presented above, EZH2 expression is upregulated in suprabasal keratinocytes. However, silencing it led to premature expression of differentiation genes, *KRT1* and *LOR* while overexpressing it did not inhibit Ca^{2+}-induced differentiation. Based on these findings, it was investigated whether these different observations were due to EZH2 having different functional roles in undifferentiated basal and differentiated suprabasal epidermal keratinocytes, NHEKs transfected with siRNA targeting EZH2 together with a small molecule EZH2 inhibitor GSK126 were induced to differentiate by high Ca^{2+} condition 24 hours post transfection and treatment while another set of NHEKs induced to differentiate by high Ca^{2+} condition were transfected and treated with siRNA and GSK126 24 hours post induction of differentiation (Fig.3.2.6A). Transfection efficiency and gene expression was analysed by RT-qPCR and Western blot analyses.

RT-qPCR analysis showed transfection efficiency of about 75 – 80% in both differentiated and undifferentiated keratinocytes (Fig. 3.2.6B). In addition, a significant upregulation in the expression of terminal differentiation associated genes: *KRT1*, *KRT10*, *LOR*, *FLG* and the lncRNA *TINCR* was detected in EZH2 depleted undifferentiated keratinocytes compared to EZH2-depleted differentiated keratinocytes (Fig. 3.2.6C), suggesting that

EZH2 depletion does not stimulate further differentiation in already differentiating keratinocytes.

Ca^{2+}-induced differentiation was confirmed by the increased expression of K10 protein level in both undifferentiated and differentiated keratinocytes, however, its expression was markedly increased in undifferentiated EZH2 depleted keratinocytes (Fig. 3.2.6D). In addition, EZH2 protein level was observed reduced in EZH2 depleted differentiated and undifferentiated keratinocytes with a corresponding reduction in H3K27me3 level (Fig. 3.2.6D). Interestingly, a higher EZH2 protein level was observed in differentiated keratinocytes transfected with a non-targeting siRNA compared to corresponding undifferentiated keratinocytes (Fig. 3.2.6B and D), confirming earlier data showing EZH2 highly expressed in differentiating keratinocytes.

Taken together, these results suggest that EZH2 prevents the premature expression of terminal differentiation associated genes in undifferentiated basal keratinocytes and modulates the dynamics of differentiation in differentiated suprabasal keratinocytes.

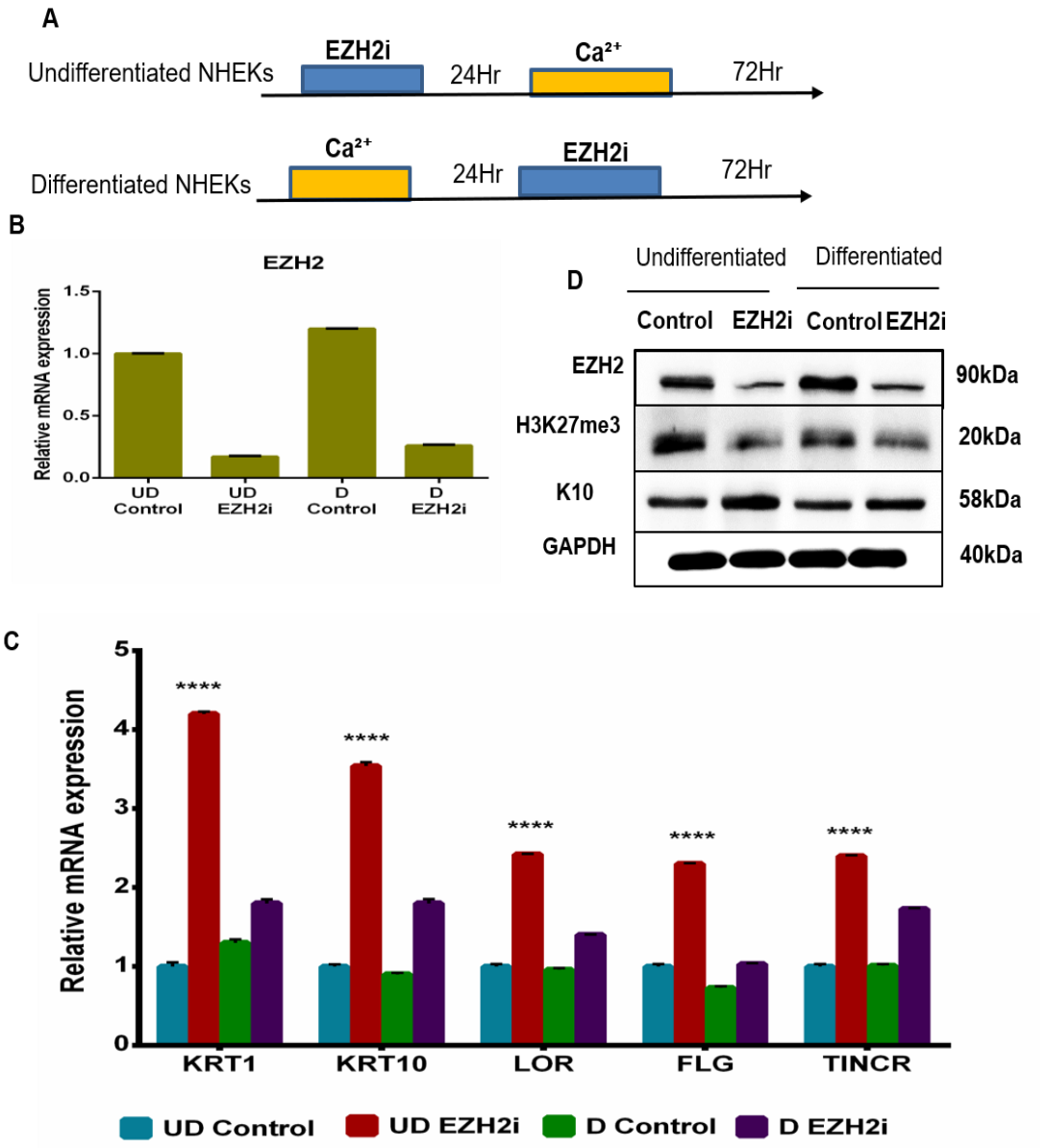


Figure 3.2.6. EZH2 prevents the premature expression of terminal differentiation associated genes in undifferentiated basal keratinocytes and modulates the dynamics of differentiation in differentiated suprabasal keratinocytes (A) Schematic diagram showing EZH2 silencing in a model of undifferentiated and differentiated NHEKs (B) RT-qPCR showing EZH2 expression after EZH2 silencing in differentiated and undifferentiated NHEKs (C) RT-qPCR showing significant upregulation of terminal differentiation genes *KRT1, KRT10, LOR, FLG* and the lncRNA *TINCR* in EZH2 depleted undifferentiated vs differentiated NHEKs (Mean±SEM; n=4, ****P<0.0001) (D) Western blot

showing expression of EZH2, H3K27me3 and K10 in EZH2 depleted undifferentiated vs differentiated NHEKs. GAPDH was used as a loading control. UD - Undifferentiated NHEKs, D - Differentiated NHEKs, Control - Control siRNA, EZH2i - EZH2 siRNA+GSK126

3.2.7 EED knockdown leads to the early expression of differentiation associated genes in NHEKs

EED, SUZ12 and EZH1/2 are core subunits of PRC2. Several lines of evidence suggest that EZH2 needs to form a complex with EED and SUZ12 for it to effectively carryout its HMTase function (Brooun et al., 2016; Cao and Zhang, 2004). Based on these findings, the functional role of EED in keratinocyte differentiation was assessed; NHEKs were transfected with siRNA targeting EED. Transfection efficiency and gene expression were analysed by RT-qPCR analysis.

RT-qPCR analysis confirms transfection efficiency and revealed a downregulation of *EZH1* and *EZH2* expression after EED knockdown (Fig. 3.2.7A-C), suggesting destabilisation of *EZH1/2* transcripts as has been shown in other cell types (Kim et al., 2013). Furthermore, a downregulation of *KRT1* and upregulation of *LOR*, *FLG* and the lncRNA *TINCR* was observed in EED depleted keratinocytes compared to keratinocytes transfected with the non-targeting siRNA (Fig. 3.2.7D), suggesting that silencing EED induces the premature expression of these differentiation associated genes.

Taken together, these results suggest that EED, like its other PRC2 component, EZH2 also controls epidermal differentiation by preventing the premature expression of differentiation associated genes in basal keratinocytes.

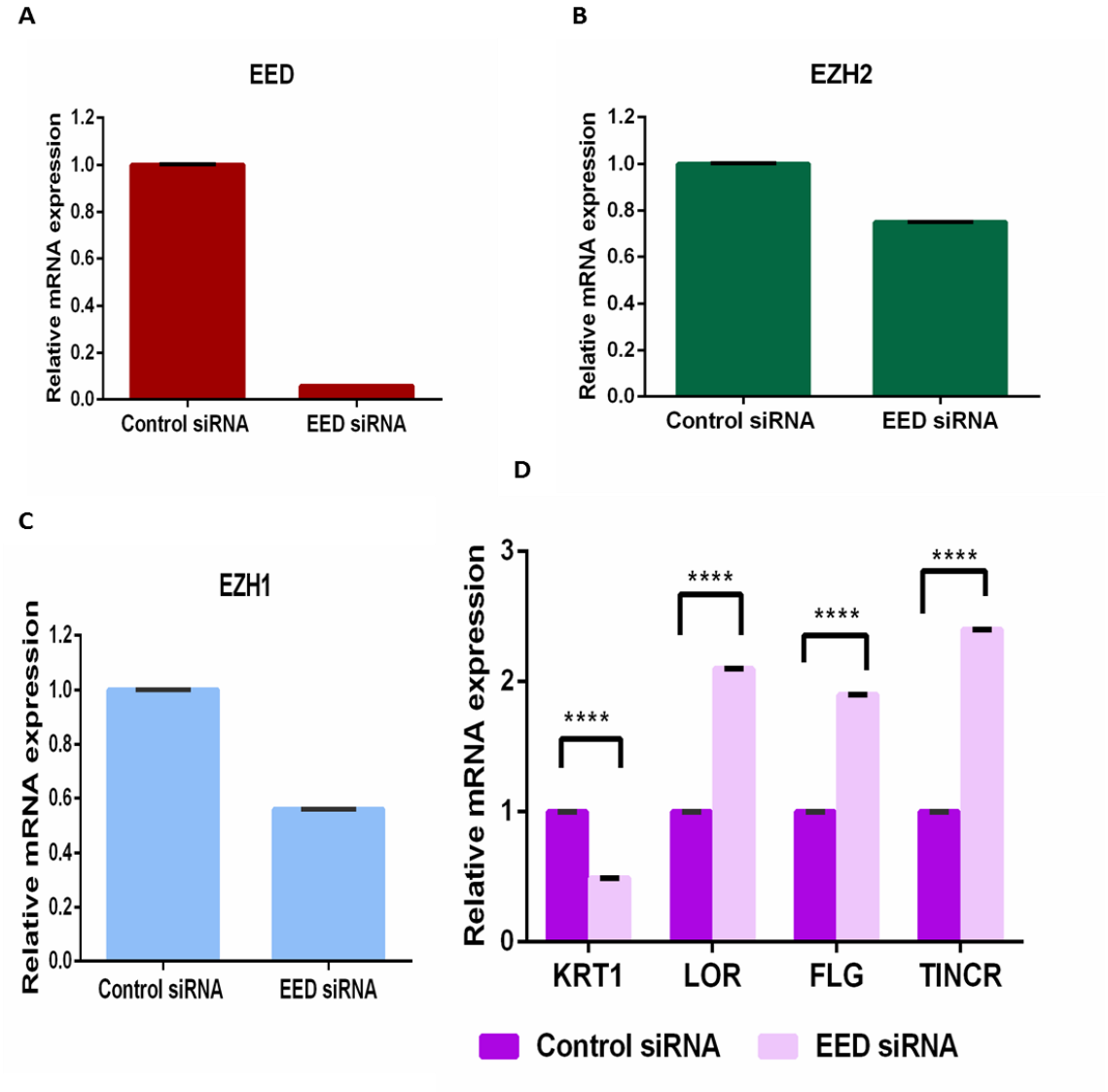


Figure 3.2.7. EED knockdown leads to the early expression of differentiation associated genes in NHEKs (A) RT-qPCR confirming knockdown of EED in NHEKs (B-C) RT-qPCR showing the expression of EZH2 and EZH1 in EED deficient NHEKs (D) RT-qPCR showing downregulation of *KRT1* expression *and* upregulation of *LOR, FLG* and the lncRNA *TINCR* in EED deficient NHEKs (Mean±SEM, n=3, ****P<0.0001). NHEKs – Normal Human Epidermal Keratinocytes

3.2.8 Inhibition of both EZH1 and EZH2 does not alter the expression of terminal differentiation genes in NHEKs

There is evidence that EZH1 can compensate for the loss of EZH2 (Ezhkova et al., 2011; Ezhkova et al., 2009). However, EZH1 is shown to be a weaker methyltransferase compared to EZH2 (Margueron et al., 2008). To begin to understand the molecular mechanisms involved in the role of EZH2 in epidermal homeostasis, we utilised small molecule inhibitors for EZH2 – GSK126, for both EZH1 and EZH2 – UNC1999 and an analogue designed as a control – UNC2400. The effect of the inhibition on terminal differentiation of keratinocytes was investigated by analysing the expression of genes implicated in the control of cell proliferation, terminal differentiation, apoptosis and senescence.

RT-qPCR analysis revealed downregulation of *EZH1* and *EZH2* expression in keratinocytes treated with UNC1999; and downregulation of *EZH2* expression with an upregulation of *EZH1* expression in keratinocytes treated with GSK126 compared to the keratinocytes treated with UNC2400 (control) (Fig. 3.2.8A). In addition, no significant changes in *KRT1*, *LOR* and *FLG* expression was detected in UNC1999 treated keratinocytes while upregulation in *KRT1*, *LOR* and *FLG* expressions was detected in GSK126 treated keratinocytes compared to the UNC2400 treated keratinocytes (Fig. 3.2.8B) suggesting that treatment with UNC1999 has no effect on the expression of differentiation genes.

Furthermore, UNC1999 treatment of NHEKs resulted in a non-significant downregulation in the expression of cell cycle inhibitor $p14^{ARF}$, non-significant

upregulation in the expression of *p19*^{INK4D}, a cycle cell inhibitor and no change in the expression of *p16*^{INK4A}, a marker of senescence and cell cycle inhibitor compared to the UNC2400 treated NHEKs while GSK126 treatment of NHEKs resulted in a non-significant downregulation of *p14*^{ARF} expression with a non-significant upregulation in the expression of *p19*^{INK4D} and *p16*^{INK4A} (Fig. 3.2.8C).

In addition, cell proliferation was assessed in NHEKs treated with the small molecule inhibitors utilising immunostaining for the mitotic cell marker H3S28p. Immunofluorescence analysis and quantification revealed a significant reduction in the percentage of H3S28p positive mitotic keratinocytes in both UNC1999 and GSK126 treated keratinocytes compared to UNC2400 treated keratinocytes (Fig. 3.2.8 D-E).

Taken together, these results suggest that inhibition of both EZH1 and EZH2 does not alter the expression of genes involved in terminal differentiation, apoptosis and senescence but reduces proliferation in NHEKs.

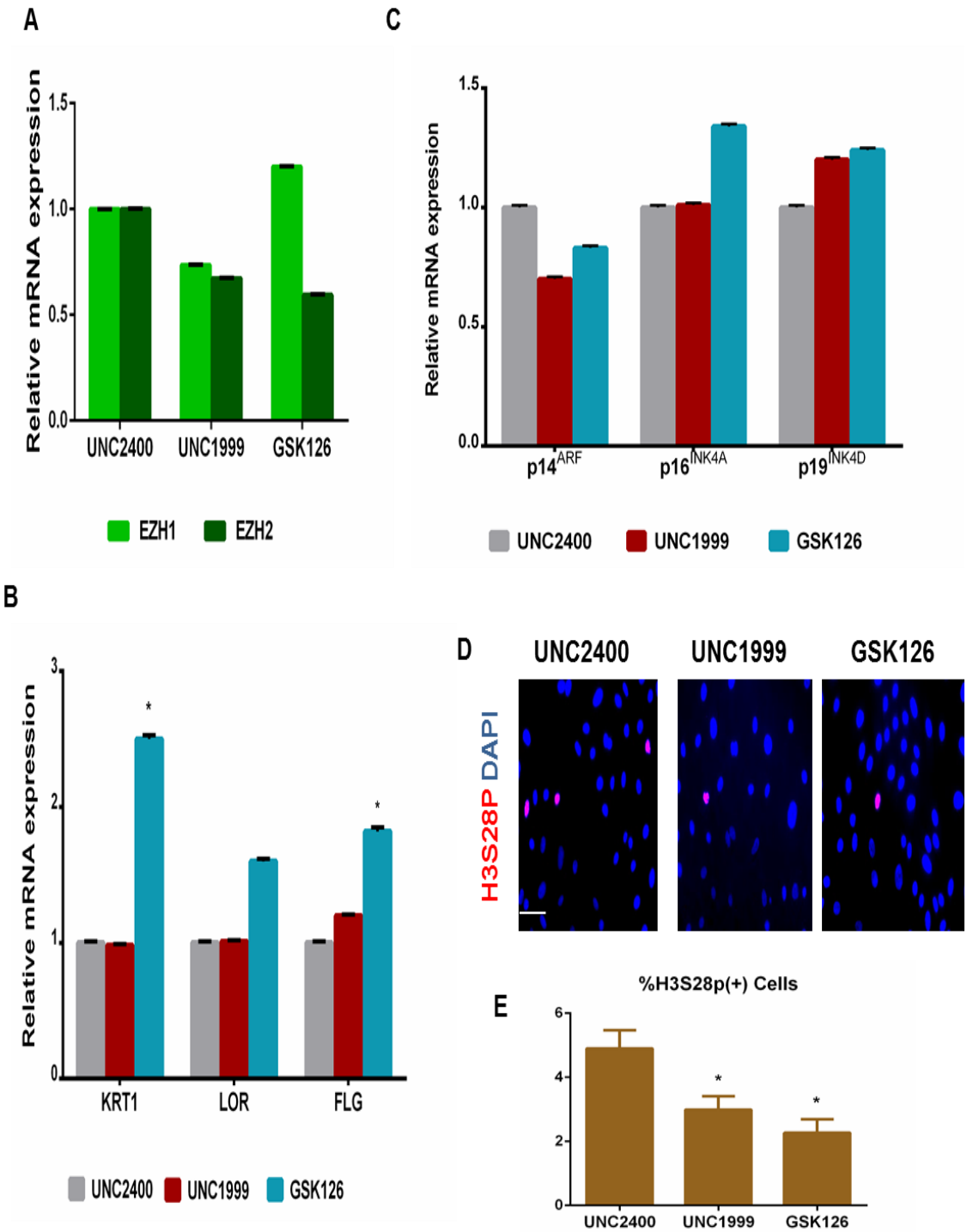


Figure 3.2.8. Inhibition of both EZH1 and EZH2 does not alter the expression of terminal differentiation associated genes in NHEKs (A) RT-qPCR showing the expression of EZH1 and EZH2 in NHEKs treated with UNC2400 (control), UNC1999 (EZH1and EZH2 inhibitor)

and GSK126 (EZH2 inhibitor) (B) RT-qPCR showing upregulation of *KRT1, LOR and FLG* expression in only GSK126 treated NHEKs compared to the UNC2400 treated NHEKs (Mean±SEM, n=3, *P<0.05) (C) RT-qPCR showing a not significant downregulation of $p14^{ARF}$ in both UNC1999 and GSK126 treated NHEKs, not significant upregulation of $p19^{INK4D}$ expression in both UNC1999 and GSK126 treated NHEKs with no change in $p16^{INK4A}$ expression in UNC1999 treated NHEKSs but a not significant upregulation of $p16^{INK4A}$ expression in GSK126 treated NHEKs. (D) H3S28p staining showing reduced proliferation in NHEKs treated with UNC1999 and GSK126 compared to NHEKs treated with UNC2400 (E) Quantification of percentage of H3S28p(+) NHEKs (Mean±SD; n=5, *P<0.05). Scale bars: 100μm. NHEKs – Normal Human Epidermal Keratinocytes

3.2.9 Loss of EZH2 leads to reduced proliferation and induces apoptosis in NHEKs

To further understand the functional role of EZH2 in epidermal homeostasis, the effect of EZH2 depletion on keratinocyte proliferation and apoptosis was investigated, NHEKs were transfected with siRNA targeting EZH2 followed by analysis of proliferation and apoptosis by utilising 5-bromo-2'-deoxyuridine (BrdU) assay that is based on BrdU incorporation into cellular DNA during cell proliferation and activated caspase 3 detection utilising anti-BrdU and anti-Caspase 3a antibodies.

Immunofluorescence analysis and quantification revealed a significant reduction in the percentage of BrdU positive proliferating keratinocytes and a significant increase in the percentage of activated Caspase 3 positive apoptotic keratinocytes in EZH2 depleted keratinocytes compared to the corresponding control (Fig. 3.2.9A-D), suggesting that EZH2 is required for proliferation and EZH2 depletion activates apoptosis.

Taken together, these results suggest that loss of EZH2 leads to reduced proliferation and induced apoptosis in NHEKs. The effect on proliferation is similar to the negative effect on proliferation observed earlier in EZH1 and EZH2 depleted NHEKs.

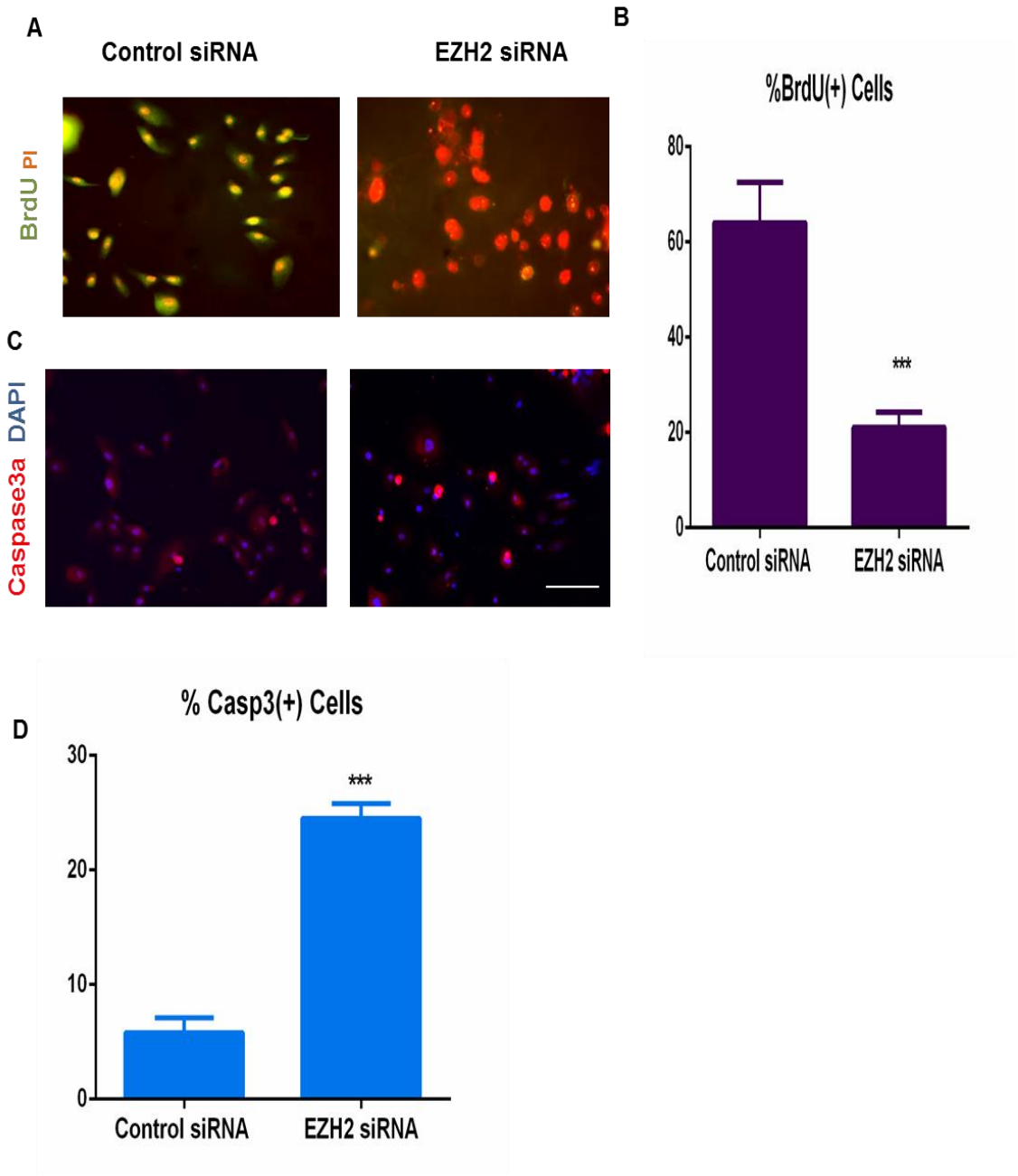


Figure 3.2.9. Loss of EZH2 leads to reduced proliferation and induced apoptosis in NHEKs (A) BrdU staining showing reduced proliferation in EZH2 deficient NHEKs (B) Quantification of percentage of BrdU(+) NHEKs (Mean±SD; n=5, ***P<0.001) (C) Activated Caspase 3 (Caspase3a) staining showing increased apoptosis in EZH2 deficient NHEKs (D) Quantification of percentage of Casp3(+) NHEKs (Mean±SD; n=5, ***P<0.001). Scale bar: 100μm. PI - Propidium iodide

3.2.10 EZH2 expression is downregulated in aged NHEKs

Data presented above show an increase in the expression of $p16^{INK4A}$ in EZH2 depleted keratinocytes. There is evidence that $p16^{INK4A}$ expression is upregulated in aging human skin (Ressler et al., 2006). To investigate if EZH2 has any role in human skin aging and senescence, NHEKs isolated from juvenile foreskin from a single donor (Young) and adult skin pooled from two female donors aged 57yrs and 64yrs (Aged) were treated with a small molecule EZH1 and EZH2 inhibitor UNC1999, small molecule EZH2 inhibitor GSK126 and a small molecule UNC2400 as control for four days.

RT-qPCR analysis revealed a downregulation of *EZH2* expression in aged NHEKs compared to young NHEKs treated with UNC2400, UNC1999 and GSK126 with no significant change in *EZH1* expression in young NHEKs compared to aged NHEKs (Fig. 3.2.10A). In addition, over 2-fold increases in expression of senescence-associated genes $p16^{INK4A}$ and $p19^{INK4D}$ was observed in aged NHEKs compared to young NHEKs treated with UNC2400 and GSK126 with no significant change in $p14^{ARF}$ expression in aged NHEKs compared to young NHEKs in all treatment groups (Fig.3.2.10B).

Taken together, these results suggest that EZH2 is downregulated in aged NHEKs and this leads to the upregulation of the expression of senescence-associated genes $p16^{INK4A}$ and $p19^{INK4D}$.

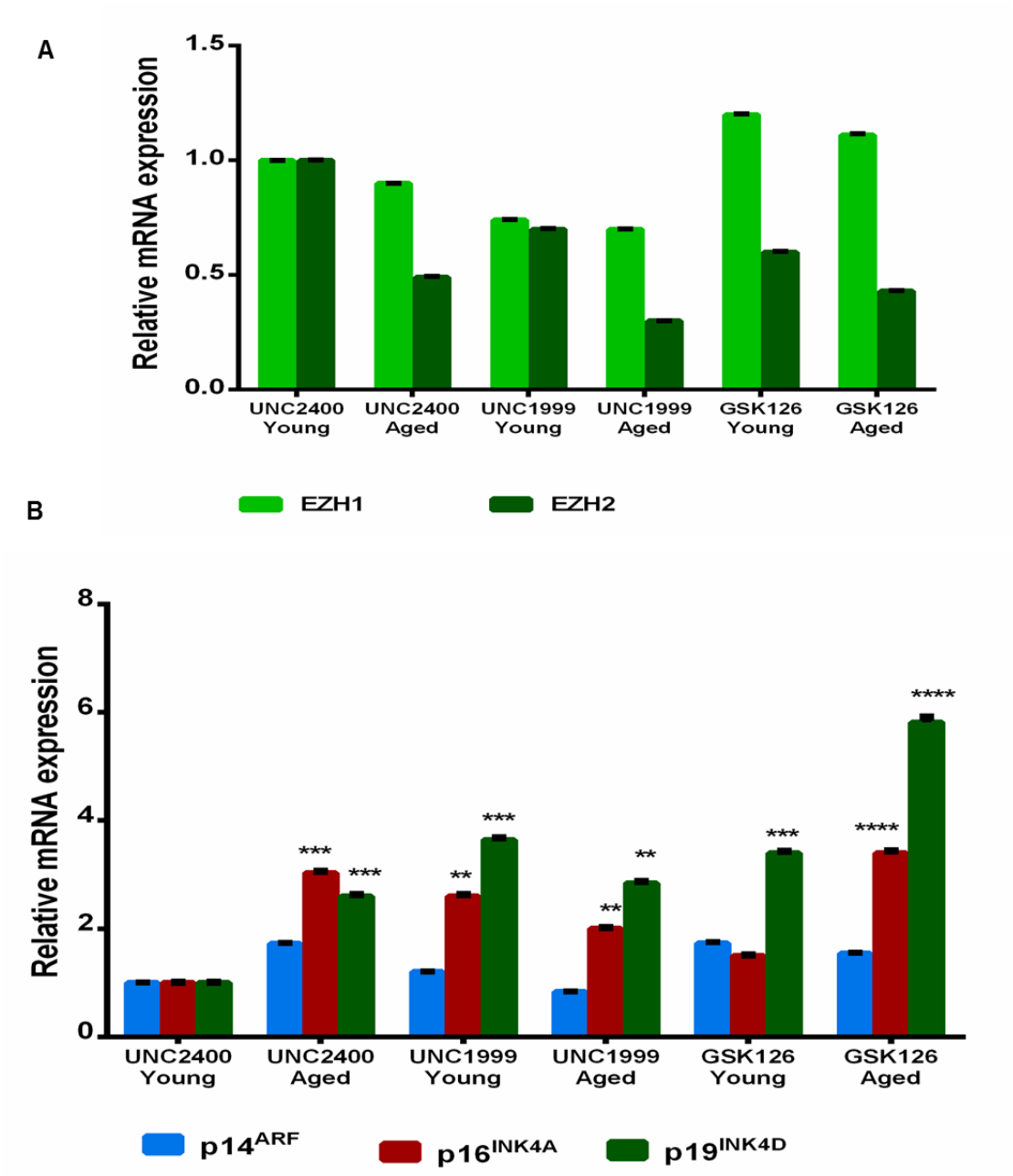


Figure 3.2.10. EZH2 is downregulated in aged NHEKs (A) RT-qPCR showing the expression of EZH1 and EZH2 in young and aged NHEKs treated with UNC2400 (control), UNC1999 (EZH1/2 inhibitor) and GSK126 (EZH2 inhibitor). (B) RT-qPCR showing significant upregulation of p16INK4A and p19INK4D expression in UNC2400, UNC1999 and GSK126 treated aged NHEKs compared to young NHEKs treated with UNC2400 (control) (Mean±SEM, n=3; **P<0.01, ***P<0.001, ****P<0.0001)

3.3 CONCLUSION

Based on the data obtained from this study, the following conclusions can be drawn:

1. In human epidermis, EZH2 is highly expressed in differentiating suprabasal keratinocytes. EZH1/2 expression and enzymatic activity increases during Ca^{2+}-induced differentiation. EZH2 plays different functional roles in basal and suprabasal layers by preventing the premature expression of terminal differentiation associated genes in undifferentiated basal keratinocytes and modulating the dynamics of differentiation in differentiated suprabasal keratinocytes.
2. Inhibition of EZH2 and EED in primary human keratinocytes *in vitro* leads to their premature differentiation by stimulating the precocious expression of differentiation-associated genes, *K1, K10, LOR, FLG and TINCR.*
3. EZH2 overexpression in primary human keratinocytes prevents the premature expression of differentiation associated genes but does not inhibit their ability to undergo Ca^{2+}-induced differentiation.
4. EZH1/2 controls the balance between proliferation and apoptosis during epidermal homeostasis.
5. Inhibition of EZH2 in epidermal keratinocytes induces complex changes in gene expression programmes, including the upregulation of genes involved in terminal differentiation, such as *FLG* and *TINCR*; and downregulation of genes involved in keratinocyte proliferation, such as CCND1.

6. EZH2 might play a role in epidermal aging by altering the expression of senescence-associated genes *p16*^{INK4A} and *p19*^{INK4D}.

Taken together, the data obtained in this study suggest that in human epidermis, PRC2 is required for effective epidermal differentiation. PRC2 also controls the balance between cell proliferation and apoptosis. PRC2 may be involved in epidermal aging. Further research should be done to clearly understand the role of PRC2 subunits in epidermal aging. This study provides an important platform for further research into the possibility of modulating PRC2 activity as a new therapeutic strategy for disorders of epidermal differentiation.

CHAPTER 4: EZH2 is required for human hair growth

4.1 Introduction

Hair exerts diverse functions in mammals. These include thermal insulation, physical protection, camouflage, excretion, sensation, social interaction, dispersion of sweat and sebum (Schneider et al., 2009). In addition, it also provides a protective niche for several stem cell populations in the skin (Alonso and Fuchs, 2003; Yang and Cotsarelis, 2010). The hair follicle is composed of concentric layers that can be grouped into the outer root sheath (ORS), the inner root sheath (IRS), and the hair shaft (HS). The inner root sheath includes the companion layer, Henle's layer, Huxley layer and the IRS cuticle while the hair shaft includes the cuticle, cortex and the medulla (Schneider et al., 2009). The cells in the hair follicle are either of epithelial origin or mesenchymal origin. The cells in the ORS, IRS, matrix and hair shaft are of epithelial origin, whereas the cells in the dermal papilla and connective tissue sheath are of mesenchymal origin (Yang and Cotsarelis, 2010). Hair follicles undergo cycles of growth (anagen), regression (catagen) and resting (telogen) phases. During hair cycling, stem cells residing in the bulge are responsible for hair regeneration (Blanpain and Fuchs, 2009; Cotsarelis et al., 1990).

A role for PRC2 in regulating murine hair follicle development and homeostasis has been demonstrated in past studies (Dauber et al., 2016;

Ezhkova et al., 2011). However, the role of PRC2 in human hair follicle development and homeostasis remains unclear. In this study, the role of PRC2 in human hair homeostasis is investigated by examining the role of EZH2 in human hair growth.

4.2 Results

4.2.1 EZH2 is expressed in progenitor cells and differentiating cells in human hair follicles

To determine a potential role for EZH2 in human hair growth, its expression pattern was assessed in human terminal anagen hair follicles by using immunofluorescence analysis.

The prominent expression of EZH2 was observed in the outer root sheath (ORS), inner root sheath (IRS) and precortex whilst a weak EZH2 expression was seen in the dermal papilla (DP) and connective tissue sheath (CTS) (Fig. 4.2.1A), suggesting that EZH2 plays a predominate role in the epithelial compartments of anagen hair follicles. In addition, EZH2 expression was co-localised with the follicular epithelial stem cell markers, such as CD200, K15 and K19. Immunofluorescence analysis revealed EZH2 co-expression with CD200 in the follicular bulge (Fig. 4.2.1B), and with K15 and K19 in progenitor cells in the ORS (Fig. 4.2.1C-D).

Taken together, these results demonstrate that EZH2 is ubiquitously expressed in the hair follicle epithelium, including progenitor cells, suggesting a potential role for EZH2 in the control of PRC2-mediated gene expression programme during human hair growth.

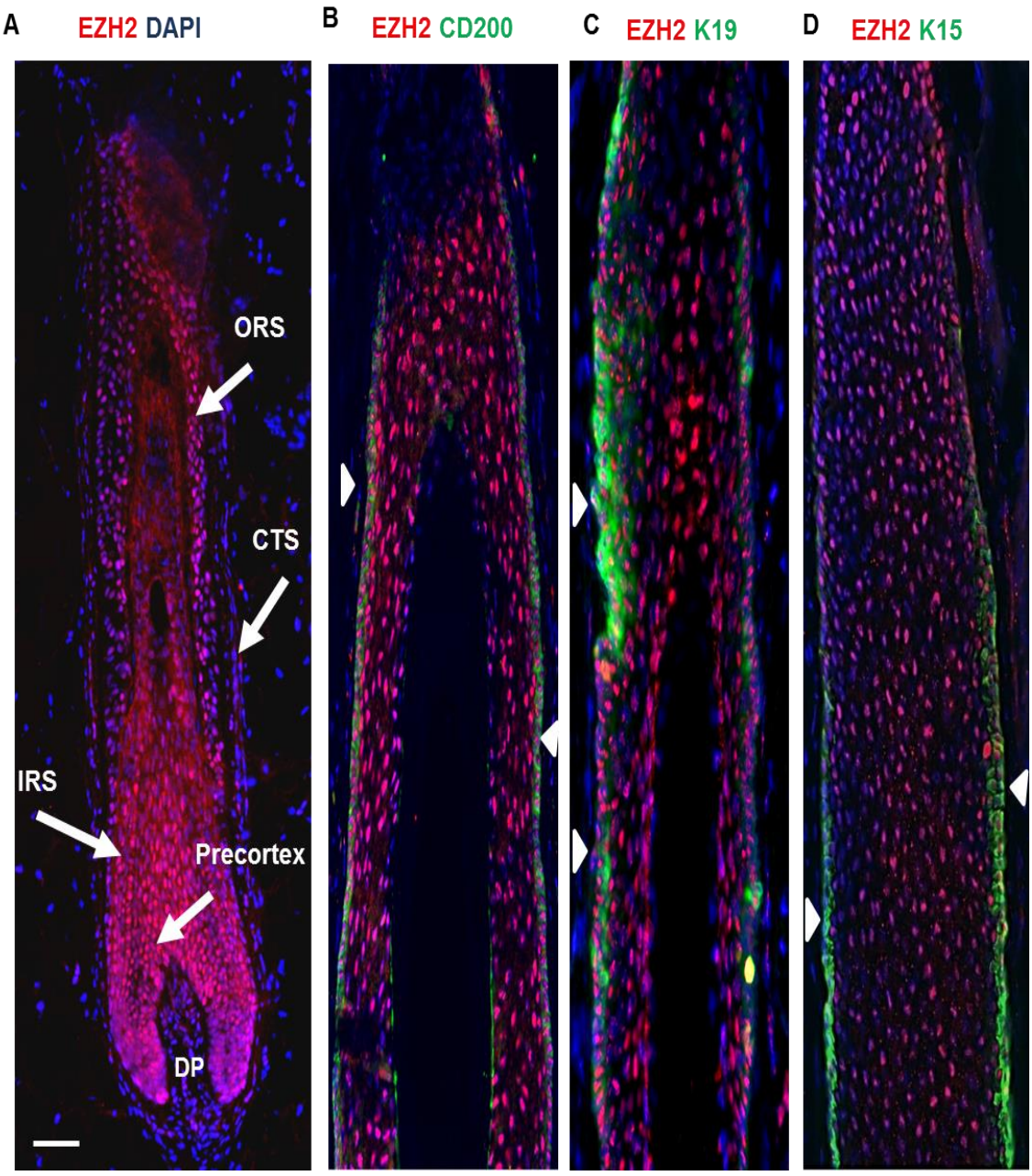


Figure 4.2.1. EZH2 is expressed in progenitor cells and differentiating cells in human hair follicles. (A) Immunofluorescence analysis of human terminal anagen hair follicle showing the prominent expression of EZH2 in the outer root sheath (ORS), inner root sheath (IRS) and precortex with weak EZH2 expression in the dermal papilla (DP) and connective tissue sheath (CTS). (B-D) Immunofluorescence analysis showing EZH2 co-expressed with CD200 in the follicular bulge (B), with K15 and K19 in progenitor cells (C-D) in the ORS. Scale bars: 50μM. ORS – Outer root sheath

4.2.2 Inhibition of EZH2 activity in human hair follicles *ex vivo* results in hair growth retardation

To investigate the functional role of EZH2 in human hair growth, activity of EZH2 was inhibited in human hair follicles *ex vivo* model utilising a selective small molecule, 3-Deazaneplanocin A (DZNep). DZNep is a S-adenosyl homocysteine hydrolase inhibitor which induces EZH2 depletion (Tan et al., 2007).

HFs were treated with either 5 or 10μM DZNep; effect of DZNep treatment on hair growth was evaluated by morphological analysis in comparison to the untreated control HFs. DZNep treatment resulted in reduced hair follicle length that was seen at day 3 and day 6 after the beginning of the experiment in a dose dependent manner compared to the untreated control (Fig. 4.2.2A-B).

In addition, assessment of proliferation and apoptosis was performed using immunohistochemical analysis of DZNep treated and untreated HFs collected at day 6. Results revealed that the number of proliferating PCNA-positive cells were significantly reduced in DZNep treated HF in a dose dependent manner compared to the untreated control (Fig. 4.2.2C). The latter was associated with an increased number of apoptotic cells marked by active form of Caspase 3 in a DZNep dose dependant manner compared to the untreated control (Fig. 4.2.2D). This suggests that the hair growth retardation observed in DZNep treated HF was as a result of reduced proliferation and increased apoptosis in the ORS.

Taken together, these results suggest that inhibition of EZH2 activity with a selective small molecule DZNep results in hair growth retardation *ex vivo* associated with reduced proliferation and increased apoptosis in the ORS.

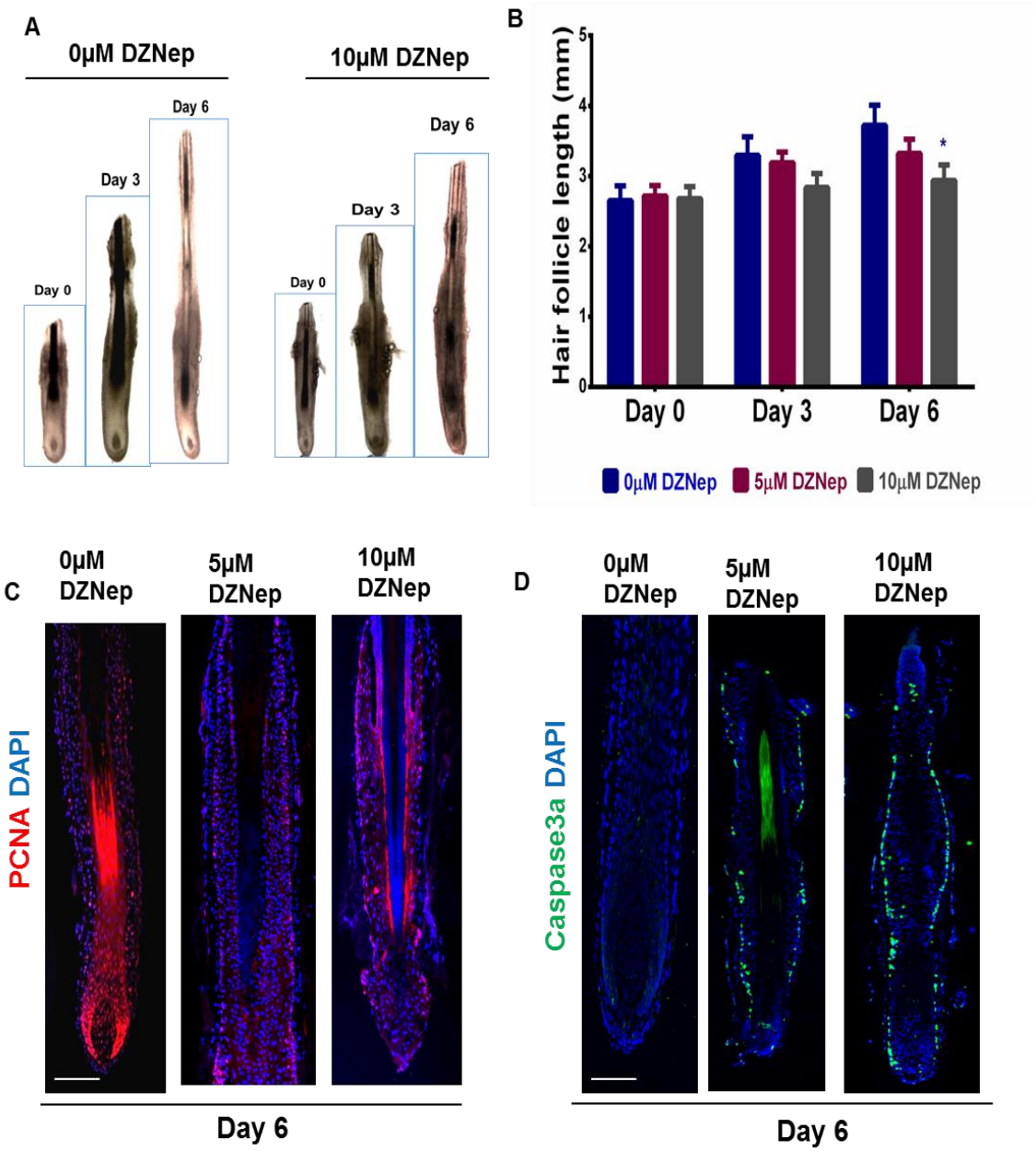


Figure 4.2.2. Inhibition of EZH2 activity in human hair follicles *ex vivo* results in hair growth retardation. (A-B) Inhibition of EZH2 activity with a selective small molecule, DZNep, causes a dose-dependent retardation of hair growth *ex vivo.* (Mean±SD, n= 3 donors and 6 HFs for each treatment group, P*<0.05) (C) Immunohistochemical staining with anti-PCNA antibody showing a dose-dependent decrease in proliferation (D) Immunohistochemical staining with anti-Caspase 3a antibody showing a dose-dependent increase in apoptosis in the ORS. Scale bars: 50μM

4.2.3. Knockdown of EZH2 expression in human hair follicles *ex vivo* accelerates anagen to catagen transition

To further investigate the functional role of EZH2 in hair growth, EZH2 expression was knockdown in human hair follicle *ex vivo* model by utilising siRNA against EZH2.

Effect of EZH2 siRNA on anagen-catagen transition was evaluated by morphological analysis. At day 7, about 38% of the HFs in EZH2 silenced group entered catagen compared to 13% in the control group (Fig. 4.2.3A-B).

The efficiency of silencing was evaluated by RT-qPCR and revealed that 40% downregulation of *EZH2* transcript was achieved (Fig. 4.2.3C). In addition, the effect of EZH2 silencing on proliferation and the expression of stem cell markers was assessed by RT-qPCR. The results revealed that the expression of the proliferation marker *PCNA* was significantly downregulated, stem cell markers *CD200* and *K15* were downregulated though not significant while the expression of stem cell marker *K19* was significantly upregulated in EZH2 deficient HFs (Fig. 4.2.3D-E).

Taken together, these results demonstrate that *EZH2* silencing in hair follicle *ex vivo* accelerates anagen-catagen transition. These data confirm that EZH2 is required for keratinocyte proliferation and might be involved in the regulation of stem cell activity in human hair follicles.

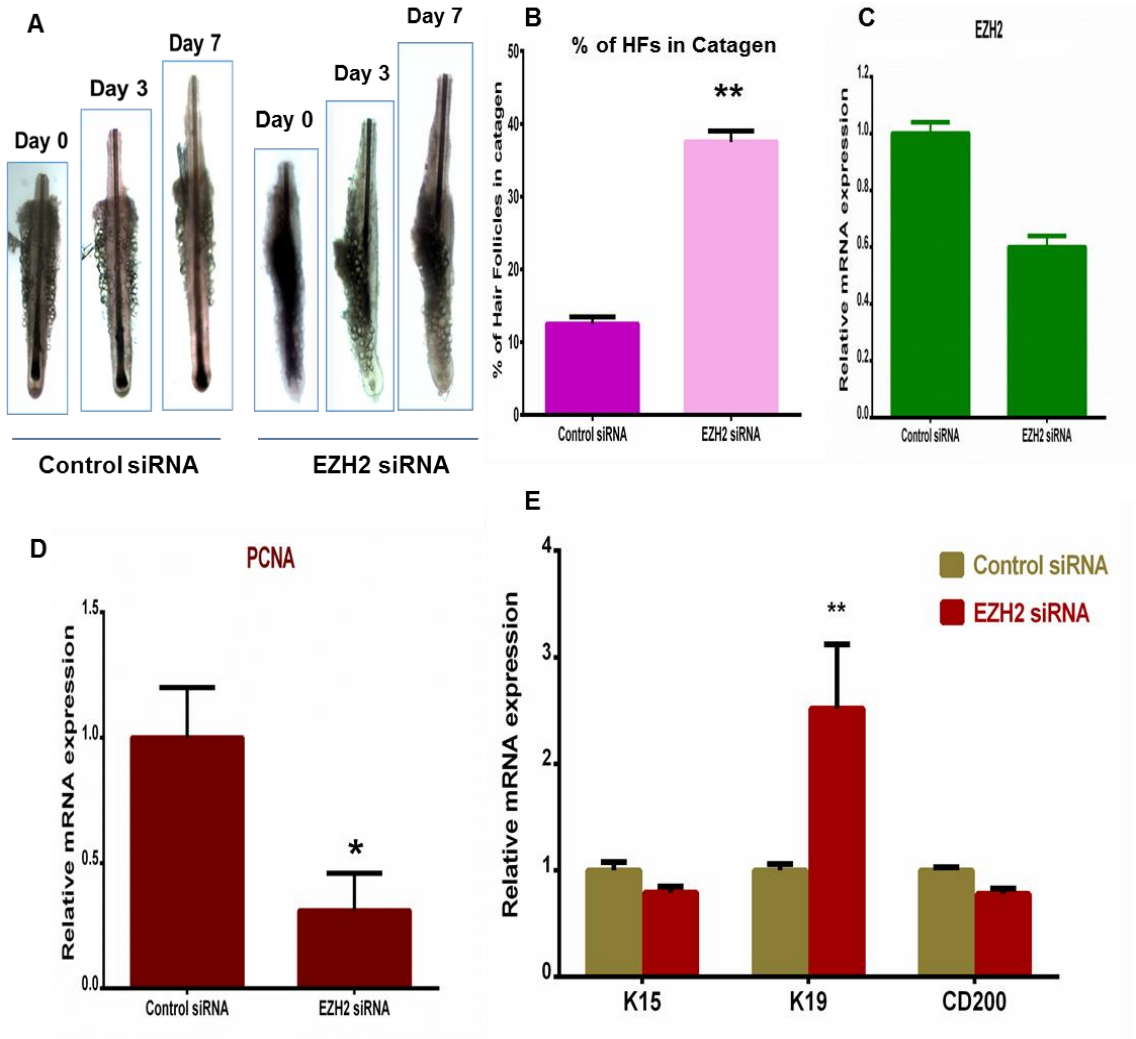


Figure 4.2.3. Knockdown of EZH2 expression in human hair follicles *ex vivo* accelerates anagen to catagen transition (A) Knockdown of EZH2 expression with siRNA showing accelerated transition from anagen to catagen in HF *ex vivo* model transfected with EZH2 siRNA. (B) Quantification of HFs that entered catagen 7 days post transfection (Mean±SD, n=3 donors and 6 HFs for each treatment group, **P<0.01) (C) RT-qPCR showing silencing efficiency in HFs transfected with EZH2 siRNA collected at day 7 (D) RT-qPCR showing downregulation of *PCNA* expression in HF transfected with EZH2 siRNA compared to the control (Mean±SD, n=3, *P<0.05) (E) RT-qPCR showing down regulation of *K15* and *CD200* expression with significant upregulation of *K19* expression in EZH2 depleted HFs compared to the control (Mean±SD, n=3, **P<0.01). HFs – Hair follicles.

4.2.4. Knockdown of EZH2 reduces proliferation and alters the expression of stem cell markers in ORSKs

To further investigate EZH2 role in the regulation of hair growth, keratinocytes isolated from the ORS of human HFs (ORSKs) were transfected with EZH2 siRNA. Silencing efficiency was confirmed by RT-qPCR that demonstrated reduced levels of *EZH2* transcript in EZH2 siRNA versus scrambled siRNA transfected ORSKs (Fig. 4.2.4A). The effect of EZH2 silencing on keratinocytes proliferation was investigated by analysing the expression of genes implicated in the control of cell proliferation, apoptosis and senescence.

Suppressed expression of EZH2 caused a significant downregulation of the expression of proliferation marker *PCNA* compared to the control (Fig. 4.2.4B), consistent with earlier findings in NHEKs were EZH2 silencing led to their reduced proliferation.

In addition, the evaluation of the effects of EZH2 silencing on the expression of stem cell markers showed negative regulation of *CD200* expression, significant increase in *K15* expression, while *K19* and *CD34* expression remained unchanged in EZH2 depleted ORSKs compared to the control (Fig. 4.2.4C). Furthermore, the expressions of cell-cycle inhibitors such as *p21, $p16^{INK4A}$, $p14^{ARF}$* and *$p19^{INK4D}$* were altered. Expression of *p21* was downregulated though not significant and *$p16^{INK4A}$* was significantly downregulated while expression of *$p14^{ARF}$* was significantly upregulated with *$p19^{INK4D}$* expression also upregulated though not significant in EZH2 depleted ORSKs compared to the control.

Taken together, these results demonstrate that EZH2 regulates proliferation at least in part via the *INK4A/ARF* locus and contributes to the control of stem cell activity in ORSKs.

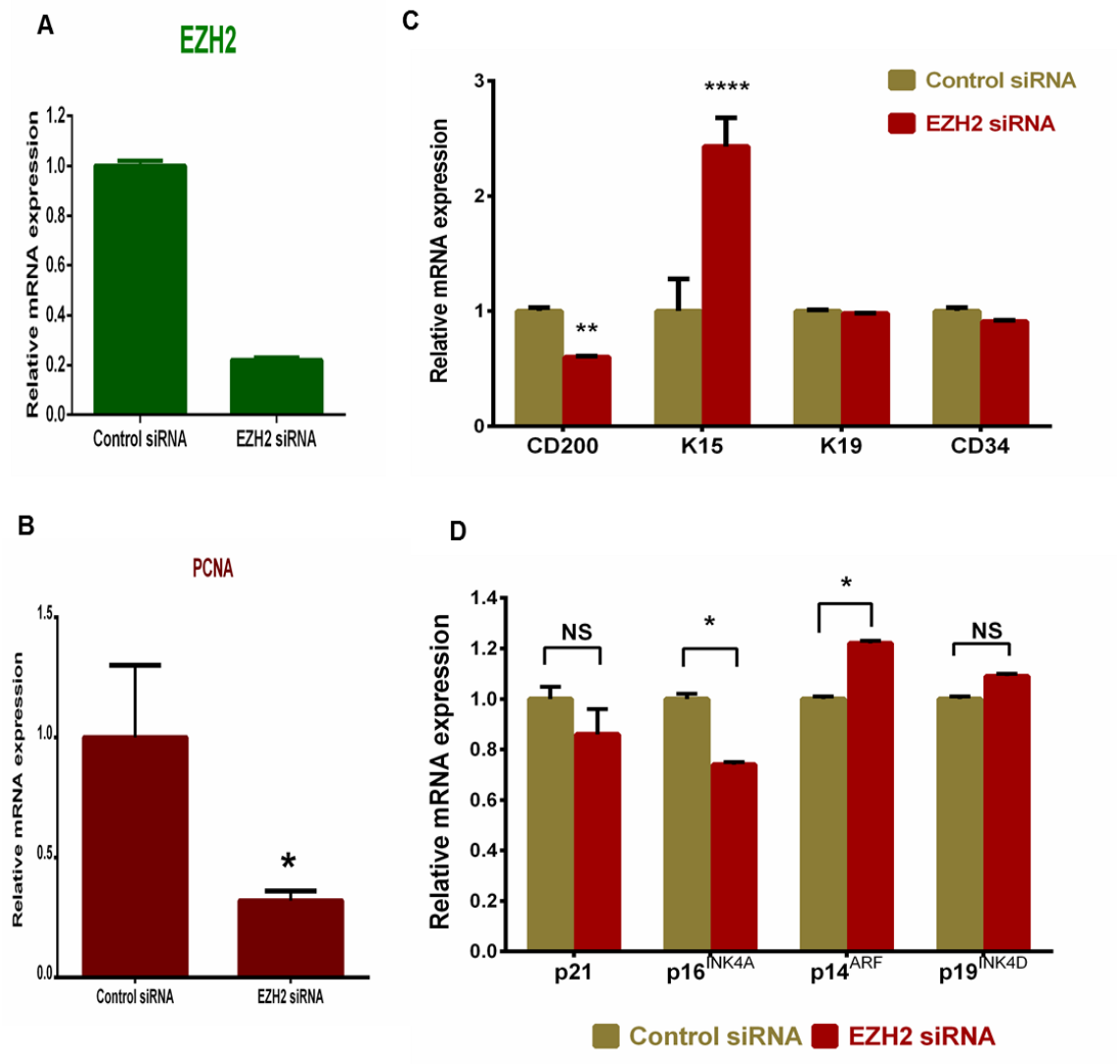


Figure 4.2.4. Knockdown of EZH2 reduces proliferation and alters the expression of stem cell markers in ORSKs (A) RT-qPCR showing knockdown efficiency in ORSKs transfected with EZH2 siRNA (B) RT-qPCR showing downregulation of proliferation marker, *PCNA* in EZH2-depleted ORSKs compared to the control (Mean±SD, n=3 (triplicates), *P<0.05) (C) RT-qPCR showing negative regulation of *CD200* expression and increase in *K15* expression while the expression of *K19* and *CD34* remains unchanged in EZH2 depleted ORSKs compared to the control (Mean± SD, n=3, **P<0.01, ****P<0.0001) (D) RT-qPCR showing expression of cell cycle regulators in EZH2 depleted ORSKs (Mean±SD, n=3 (triplicates), *P<0.005). ORSKs – Outer root sheath keratinocytes.

4.3 CONCLUSION

Based on the data obtained from this study, the following conclusions can be made:

1. PRC2 subunit EZH2 is ubiquitously expressed in epithelial cells of human anagen hair follicles. EZH2 is also expressed in follicular epithelial stem cells and progenitor cells.
2. Inhibition of EZH2 activity in human hair follicles accelerates anagen-catagen transition and retards hair growth. The hair growth retardation is associated with reduced cell proliferation and increased apoptosis in the outer root sheath.
3. Inhibition of EZH2 in outer root sheath keratinocytes (ORSKs) results in the upregulation of senescence-associated gene, $p14^{ARF}$ and stem cell marker, *K15.*

Taken together, the data obtained in this study suggest that EZH2 prolongs anagen phase by promoting cell proliferation, at least in part via repressing the INK4A/ARF locus and represses apoptosis in outer root sheath cells. This study raises an avenue for further research into the possibility of modulating EZH2 activity as a new therapeutic strategy for hirsutism and hair loss disorders.

CHAPTER 5: DISCUSSION

5.0 The Role of Polycomb Repressive Complex 2 (PRC2) in epidermal homeostasis and hair growth

In recent years, various studies have demonstrated a role for epigenetic mechanisms in mammalian skin development and homeostasis (Dauber et al., 2016; Driskell et al., 2011; Ezhkova et al., 2011; Ezhkova et al., 2009; Sen et al., 2010). For example, loss of function studies in mouse skin demonstrated a crucial role for PRC2 in regulation of proliferation, differentiation and apoptosis (Dauber et al., 2016; Ezhkova et al., 2011; Ezhkova et al., 2009). Although, PRC2 has been studied extensively in murine skin development and homeostasis, its role in human skin development and homeostasis is still not well understood. This study demonstrated that PRC2 is involved in epidermal homeostasis and hair growth by regulating keratinocyte proliferation, epidermal differentiation, keratinocyte senescence, hair cycling and apoptosis.

5.1 Changes in PRC2 activity during terminal differentiation of human epidermal keratinocytes

In this study, for the first time, it was shown that EZH2 was highly expressed in differentiating suprabasal keratinocytes with weaker expression in undifferentiated basal keratinocytes in human epidermis. EZH2 was also

observed localised in the nucleus of cultured primary human keratinocytes; however, its expression was upregulated in differentiating keratinocytes upon Ca^{2+}-induced differentiation. In addition, the expression of PRC2 subunit EZH1, a homolog of EZH2, as well as H3K27me3 levels was increased upon Ca^{2+}-induced differentiation. This data somehow contradicts with a previous study that reported an EZH2 abundant expression in undifferentiated basal keratinocytes compared to suprabasal keratinocytes in adult human epidermis (Gannon et al., 2015). In addition, Gannon et al also reported that they observed no change in EZH2 protein level upon Ca^{2+}-induced differentiation in primary human keratinocytes compared to undifferentiated proliferating keratinocytes. However, this conclusion made by Gannon et al., contradicts their own western blot data, which clearly shows a marked increase in EZH2 protein expression in Ca^{2+} treated differentiating keratinocytes compared to undifferentiated cells (Figure 1b, Gannon et al., 2015). The differences observed between the data obtained from this study and Gannon's study in EZH2 expression pattern in adult human epidermis might be due to differences in detection methods used, for example, different anti-EZH2 antibodies used for immunostaining and western blot. However, the conclusion from this study was drawn from data obtained using a wide range of methods including immunofluorescent staining, RT-qPCR of FACS-sorted and cultured primary cells and Western blot analyses (Fig. 3.2.1 and 3.2.2). Interestingly, this study for the first time revealed several notable differences in EZH2 expression pattern in adult mouse epidermis versus adult human epidermis. In contrast to human skin, Ezh2 expression waned with age in basal keratinocyte and was barely detectable in suprabasal

keratinocytes in adult mouse epidermis, as well as was downregulated upon Ca^{2+}-induced differentiation of primary mouse keratinocytes (Ezhkova et al., 2009). Based on these notable differences observed, we can speculate that EZH2 may have different functional roles in human and mouse epidermis.

For decades, elevated calcium levels have been confirmed to be a trigger for keratinocyte differentiation both *in vitro* and *in* vivo (Bikle et al., 1996; Elias et al., 2002; Hennings et al., 1980; Menon et al., 1985; Pillai et al., 1990). As H3K27me3 and EZH2 transcript and protein levels were elevated in differentiating keratinocytes both *in vivo* and *in vitro,* EZH1/2 methyltransferase activity upon Ca^{2+}-induced differentiation in human primary keratinocytes was investigated. Interestingly, the methyltransferase activity was increased by 3-fold in differentiating keratinocytes compared to undifferentiated control cells. As the increase in methyltransferase activity could solely be attributed to the increase in EZH1/2 transcripts and protein levels, future studies are required to address if the intrinsic enzymatic activity of EZH1/2 also changes in differentiating human keratinocytes. Nevertheless, a direct correlation between elevated calcium level and increased K27 tri-methylation of Histone H3 suggests that PRC2 is involved in controlling gene expression not only in the basal layer but also in suprabasal cells. Ezhkova et al. demonstrated that Ezh2 functions in mouse basal progenitors, by mediating the repressive mark H3K27me3 on epidermal differentiation genes and preventing their activation by the transcription factor AP1 (Ezhkova et al., 2009). To explain the differences in the expression pattern of EZH2 between mouse and human epidermis, we have to bear in mind that human epidermis, unlike to mouse, is much thicker

due to significantly more layers of differentiating cells in each epidermal strata. We speculate that the complexity of human epidermis requires a more broader EZH2 expression pattern to control expression of epidermal differentiation genes in a temporal and spatial manner to correctly execute the terminal differentiation programme. Increased PRC2 activity may also be required to silence genes involved in the epidermal progenitor cell maintenance and proliferation in the differentiating keratinocytes. Future studies are essential to address these questions.

5.2 PRC2 is involved in the regulation of terminal differentiation in human epidermis

The main functions of PRC2 are establishing the repressive histone mark H3K27me3 and recruiting PRC1 to chromatin leading to PcG-mediated gene repression by chromatin compaction and interfering with transcription (Cao et al., 2002; Francis et al., 2004; Min et al., 2003b). EZH2 as the methyltransferase subunit of PRC2 is at the centre of this PcG-mediated gene repression. Increasing evidence indicates that PRC2 subunits are critical for the terminal differentiation of different cell types including skeletal muscle cells (Juan et al., 2011; Mousavi et al., 2012), epidermal cells (Bardot et al., 2013; Dauber et al., 2016; Ezhkova et al., 2009) and heart cells (He et al., 2012). In this study, a role for PRC2 in regulating terminal differentiation program in human epidermis using primary human keratinocytes (Normal Human Epidermal Keratinocytes - NHEKs) as a model was defined. Using a

wide range of experimental approaches, it was demonstrated that PRC2 represses an array of genes involved in the terminal differentiation programme of primary human epidermal keratinocytes. To examine the role of PRC2 in keratinocyte differentiation, siRNA and selective small molecules were used to disrupt EZH2, EED and both EZH1/2 activities in NHEKs *in vitro*. This investigation revealed that silencing EZH2 or EED results in precocious expression of early and late differentiation genes (*KRT1, LOR and FLG)* and the differentiation-associated lncRNA *TINCR* (Kretz et al., 2013) in undifferentiated NHEKs cultured in low calcium medium. Interestingly, Ca^{2+}-induced differentiation of EZH2-depleted NHEKs leads to a significantly greater expression of terminal differentiation genes (*KRT1, LOR*, *FLG* and *TINCR)* compared to control cells. These data are consistent with earlier studies, which demonstrated that the depletion of PRC2 subunits EZH2 and SUZ12 in epidermal progenitor cells results in premature expression of epidermal differentiation-associated genes (Ezhkova et al., 2009; Sen et al., 2008). Previous studies in mouse adult epidermis suggest that EZH1 may compensate for the loss of EZH2 (Ezhkova et al., 2011; Ezhkova et al., 2009). This was tested by disrupting EZH1 and EZH2 activities in NHEKs. Interestingly, inhibition of EZH1 and EZH2 activities in NHEKs simultaneously did not have any significant effect on the expression of differentiation-associated genes compared to the control. This is consistent with previous studies done in Ezh1/2 double knockout mice, where no epidermal defects where observed in postnatal skin (Dauber et al., 2016; Ezhkova et al., 2011) suggesting that simultaneous ablation of Ezh1 and Ezh2 in mice does not accelerate epidermal differentiation or epidermal

barrier formation. EZH1/2 are the only PRC2 subunits that have methyltransferase capabilities, however, EZH1 is a weaker methyltransferase compared to EZH2 (Margueron et al., 2008; Shen et al., 2008) and PRC2-EZH1 may target different sets of genes from PRC2-EZH2 in the human epidermis. Therefore, molecular events or pathways that compensate for the loss of EZH1 and EZH2 activities in epidermal keratinocytes, though beyond the scope of this project objectives, remains to be elucidated.

EZH2-depleted keratinocytes express higher levels of differentiation associated genes (Ezhkova et al., 2009; Sen et al., 2008). Therefore, EZH2 is important in regulating keratinocyte differentiation. To test this hypothesis, the effect of overexpression of EZH2 on keratinocyte differentiation was investigated. A lentivirus was utilised to overexpress EZH2 in NHEKs and differentiation was induced. Surprisingly, EZH2-overexpressing NHEKs that were induced to differentiate under high Ca^{2+} conditions underwent differentiation similar to the control cells, suggesting that EZH2 does not block terminal differentiation in differentiating cells. Results showed 3-fold or higher changes in expression of differentiation associated genes (*KRT1, LOR, and TINCR)* in EZH2-overexpressing keratinocytes versus the control keratinocytes upon Ca^{2+}-induced differentiation, similar to EZH2-depleted NHEKs upon Ca^{2+}-induced differentiation. This data is contrary to observations in various cancers where overexpression of EZH2 is associated with inhibition of differentiation. There is evidence that all three core PRC2 subunits are overexpressed in small cell lung cancer (Sato et al., 2013) and colorectal cancer (Liu et al., 2015). We are tempted to speculate that EZH2

overexpression in NHEKs without simultaneous overexpression of the other core subunits of PRC2, EED and SUZ12, renders PRC2-mediated repression of differentiation genes ineffective in NHEKs.

By employing microarray approach, gene expression profiles were compared between EZH2-depleted NHEKs and NHEKs transfected with a non-targeting control. As expected, depletion of EZH2 resulted in a higher percentage of gene upregulation in NHEKs. The microarray and RT-qPCR analyses showed changes in expression of genes involved in the control of keratinocyte differentiation, epidermis development, keratinocyte proliferation, cell cycle arrest and WNT signalling. Importantly, EZH2-depleted keratinocytes showed marked increase in expression of genes involved in keratinocyte differentiation such as *TINCR, FLG, FOXN1, KRT13* and *RPTN,* while the expression of *CCND1,* involved in keratinocyte proliferation, was decreased. This confirms the differentiated status of EZH2-depleted keratinocytes.

Previous studies reported that EZH2 is highly expressed in proliferative cells and reduced in differentiating cells in mouse epidermis and other tissues (Ezhkova et al., 2009; Margueron et al., 2008). However, data obtained from this study has confirmed EZH2 highly expressed in differentiating cells of the human epidermis both *in vitro and in vivo.* We speculate that EZH2 has different functional roles in basal and suprabasal keratinocytes in human epidermis. This hypothesis was tested by developing an *in vitro* model for studying the effects of EZH2 depletion in undifferentiated basal and differentiated suprabasal keratinocytes. In this model, differentiation was induced in EZH2-depleted NHEKs and EZH2 was depleted in NHEKs

already differentiating under high Ca^{2+} conditions. Interestingly, analysis of depletion of EZH2 in undifferentiated keratinocytes versus differentiated keratinocytes showed 2.5-fold or higher expression of differentiation-associated genes (*KRT1, KRT10, FLG, LOR* and *TINCR)* in undifferentiated keratinocytes compared to the differentiated keratinocytes. This result suggests that EZH2 depletion in undifferentiated basal keratinocytes stimulates and accelerates terminal differentiation, while EZH2 depletion in differentiating suprabasal keratinocytes neither stimulates nor accelerates their differentiation. These findings suggest a role for EZH2 in repressing the expression of both early and late epidermal differentiation genes in basal and suprabasal layers (spinous and granular) by modulating the timing of their expression to ensure an effective terminal differentiation programme.

Taken together, these results demonstrate that PRC2-mediated regulation of epidermal differentiation genes is important for an effective terminal differentiation programme in human epidermis.

These findings provide an important background for research to evaluate the possibility of modulating PRC2 activity as a therapeutic strategy for human skin diseases characterized by defective or abnormal epidermal differentiation such as atopic dermatitis and psoriasis in future.

5.3 PRC2 controls proliferation in primary human keratinocytes

Previous studies in mice demonstrated that PRC2 core subunits control the proliferative potential of basal progenitors (Dauber et al., 2016; Ezhkova et al., 2009), hepatic progenitors (Aoki et al., 2010), subventricular zone neural stem cells (Hwang et al., 2014) and skeletal muscle stem cells (Juan et al., 2011). A recent study demonstrated that EZH2 promotes the proliferation of human embryonic stem cells (Collinson et al., 2016). Microarray data obtained in this study, showed downregulation of genes involved in keratinocytes proliferation in EZH2 depleted NHEKs. Based on these findings, the effect of loss of PRC2 on keratinocyte proliferation was investigated by utilising siRNA and selective small molecules to inhibit EZH2 alone or target both EZH1 and EZH2 activities in NHEKs. The analysis of the effect of EZH1/2 silencing revealed a significant reduction in the number of proliferative BrdU-positive cells and H3S28p-positive mitotic cells, as well as a significant increase in the number of apoptotic activated Caspase 3-positive cells. These data are consistent with previous studies that demonstrated a role for PRC2 in regulating cellular proliferation (Aoki et al., 2010; Collinson et al., 2016; Dauber et al., 2016; Ezhkova et al., 2009; Hwang et al., 2014). However, Ezhkova et al. reported epidermal hyperproliferation in Ezh1/2 double knockout mice (Ezhkova et al., 2011), which contrasted with results obtained in this study in EZH1/2-depleted NHEKs where reduced proliferation was observed. Their investigation was *in vivo* while this study's investigation was *in vitro,* this could be the reason for

the observed differences. Culture conditions could also be a contributing factor in the observed differences.

This study also examined if the reduced proliferation and increased apoptosis resulted from the activation of the INK4/ARF locus, which is up-regulated by loss of PRC2 in mouse hair follicle stem cells (Dauber et al., 2016; Ezhkova et al., 2011). Although, the results showed that the expression of $p16^{INK4A}$ was upregulated about 1.4-fold in EZH2-depleted NHEKs. Overall the changes observed in the expression of $p14^{ARF}$, $p16^{INK4A}$ and $p19^{INK4D}$ in EZH1/2-depleted NHEKs were minimal compared to the control. Therefore, even though there is evidence for the regulation of the INK4/ARF locus in murine keratinocytes by PRC2 (Dauber et al., 2016; Ezhkova et al., 2009), this data does not demonstrate this in human keratinocytes. We speculate that an alternative pathway, which does not involve the INK4/ARF locus, is activated in PRC2-depleted keratinocytes that regulates proliferation. It will be interesting to investigate the genes and pathways that are regulated by PRC2 during keratinocytes proliferation in human epidermis in future research.

Taken together, these results demonstrate a role for PRC2 in regulating human keratinocyte proliferation, though not via the INK4/ARF locus.

5.4 EZH2 expression is downregulated in aged NHEKs

Senescence is associated with aging in cells. There is evidence that EZH2 expression is downregulated in senescent and aged cell (Agherbi et al., 2009; Bracken et al., 2007; Kamminga et al., 2006). The role of PRC2 in epidermal aging was examined by disrupting EZH1/2 and EZH2 activities in keratinocytes isolated from a young individual and keratinocytes isolated from older individuals. RT-qPCR analysis revealed a downregulation of EZH2 in keratinocytes from the older individuals compared to younger donor with minimal changes in EZH1 expression in both young and aged groups. The latter, suggests that EZH2 expression and not EZH1 is downregulated in aged NHEKs. This data is consistent with previous studies that have observed EZH2 expression downregulated in aged or senescent cells compared to young cells (Agherbi et al., 2009; Bracken et al., 2007; Kamminga et al., 2006; Kheradmand Kia et al., 2009; Tang et al., 2004). Increased expression of $p16^{INK4A}$ and $p19^{ARF}$/$p14^{ARF}$ is associated with senescence and there is increasing evidence that suggests that INK4/ARF expression correlates with aging in various tissues in human and mouse (Hilpert et al., 2014; Krishnamurthy et al., 2006; Liu et al., 2009; Ressler et al., 2006). The expression of members of the INK4/ARF locus was examined in EZH1/2 and EZH2 depleted young and aged keratinocytes. RT-qPCR analysis revealed a marked upregulation of $p16^{INK4A}$ and $p19^{INK4d}$ with minimal changes in $p14^{ARF}$ expression in control and EZH2-depleted aged NHEKs compared to their young counterparts, which suggests that the increased expression of $p16^{INK4A}$ and $p19^{INK4d}$ in aged NHEKs correlates with EZH2 downregulation in these aged keratinocytes. This is consistent with

previous studies that demonstrated a correlation between downregulation of BMI-1, a PRC1 subunit that has a similar expression pattern in human epidermis and cancer as EZH2 (Kim et al., 2004; Lee et al., 2008; Reinisch et al., 2007; Vonlanthen et al., 2001), and increased $p16^{INK4A}$ expression in primary human keratinocytes from older donors (Cordisco et al., 2010) and aged human skin (Ressler et al., 2006). Previous studies in mice have also demonstrated that $p19^{INK4d}$ expression is upregulated in mice during aging (Hilpert et al., 2014). Further work needs to be done to better understand the involvement of EZH2 and other subunits of PRC2 in epidermal aging.

Taken together, these results suggest that EZH2 is downregulated in aged primary human keratinocytes and prevents keratinocyte senescence by regulating the expression of $p16^{INK4A}$ and $p19^{INK4d}$.

5.5 EZH2 is expressed in human hair follicles

Previous data obtained in this study confirmed the expression of EZH2 in human epidermis. Based on this, EZH2 expression pattern was examined in human hair follicles (HFs) by immunohistochemical analysis. This investigation showed for the first time that in human anagen HF, EZH2 is ubiquitously expressed. However, its expression was more prominent in cells of the ORS, IRS and precortex than in cells of the DP and CTS.

A previous study demonstrated that human HF bulge keratinocytes enriched in hair follicle stem cells (HFSCs) are $CD200^{+}/K15^{+}/K19^{+}$ (Rittié et al., 2009), EZH2 expression was examined in these HFSCs. Immunohistochemical

analysis showed EZH2 is co-expressed with CD200 in the follicular bulge and with K15 and K19 in progenitor cells in the ORS suggesting a role for EZH2 in regulating the activity of HFSCs.

Taken together, these results confirm EZH2 expression in progenitor and differentiating cells of human anagen HFs. Based on these findings, the role of EZH2 in human hair growth was investigated in this project.

5.6 EZH2 is required for human hair follicle growth

There is evidence that suggests a fundamental role for PRC2 in HF development and homeostasis (Dauber et al., 2016; Ezhkova et al., 2011). Ezhkova et al demonstrated the importance of Ezh1 and Ezh2 in mouse HF homeostasis (Ezhkova et al., 2011). In this study, a role for EZH2 in regulating human hair follicle (HF) growth was defined using human hair follicle organ culture (HFOC) and primary ORS keratinocytes as models. Through knockdown experiments using siRNA and a selective small molecule inhibitor in HFOC and ORS keratinocytes as well as immunohistochemical and RT-qPCR analyses, it was demonstrated that EZH2 is required for human HF growth by regulating cell proliferation and HFSCs activities. This study was limited to EZH2 due to a limited supply of human HFs. In this investigation, silencing EZH2 in human HFs resulted in hair growth retardation and promoted early transition from anagen to catagen phase, suggesting a role for EZH2 in regulating the hair cycle in humans. Further analysis showed that the hair growth retardation was due to reduced proliferation and increased apoptosis in the ORS. This is consistent with

earlier studies in mice where loss of Ezh2 led to defective development of HFs resulting from decreased proliferation and increased apoptosis in HFSCs (Dauber et al., 2016; Ezhkova et al., 2011). The expression of CD200/K15/K19 was also assessed in EZH2 depleted HFs. Notably, in EZH2 depleted HFs K19 transcript level was significantly higher compared to the control. Further work needs to be done to understand why K19 is upregulated in EZH2-depleted HFs.

In ORSKs, EZH2 depletion resulted in reduced proliferation, marked downregulation of $p16^{INK4A}$ and marked upregulation of $p14^{ARF}$ expression. This is consistent with earlier data from EZH2-depleted HFs that showed reduced proliferation and increased apoptosis. In addition, the expression of CD200 was significantly downregulated in EZH2 depleted ORSKs, while K15 expression was significantly upregulated. A previous study demonstrated that $K15^{+}$ cells were preserved while $CD200^{+}/CD34^{+}$ cells were lost in androgenetic alopecia (Garza et al., 2011). Further work needs to be done to have a clearer understanding of the role of EZH2 in PRC2-mediated regulation of HFSCs activities.

Taken together, these results suggest that EZH2 promotes human HF growth by prolonging the anagen phase and preventing apoptosis of HFSCs and progenitor cells in ORS.

In conclusion, the data obtained in this project suggests that PRC2-mediated gene repression is crucial for epidermal homeostasis and human hair growth by controlling the balance between keratinocyte proliferation, senescence, differentiation and apoptosis. Thus, further understanding of the mechanisms

controlling epidermal differentiation and epidermal stem cell activities; and their alterations in hair and skin disorders will assist in developing new therapeutic strategies targeting distinct PRC2 subunits for skin and hair disorders.

Presentations

EZH2 is required for human hair follicle growth (Oral presentation PGR Mini conference, University of Bradford, 2015)

EZH2 is required for human hair follicle growth (Poster presentation FLS R&D open Day, University of Bradford, 2015)

EZH2 is required for human hair follicle growth and epidermal differentiation (Poster presentation World Hair Research congress Miami, 2015)

EZH2 is required for human hair follicle growth and epidermal differentiation (Oral presentation PGR Mini conference, University of Bradford, 2016)

EZH2 is required for human hair follicle growth and epidermal differentiation (Poster presentation ESDR meeting, Munich, 2016)

The role of Polycomb protein EZH2 in the control of terminal differentiation in human epidermis (Oral presentation DMS/CSS Research seminar, University of Bradford, 2017)

The role of Polycomb protein EZH2 in the control of terminal differentiation in human epidermis (Oral presentation CSS John Wood Memorial Lecture, University of Bradford, 2017)

EZH2 is required for human hair follicle growth and epidermal differentiation (Poster presentation BSID meeting, Manchester, 2017)

EZH2 is required for human hair follicle growth and epidermal differentiation (Poster presentation FLS R&D open Day, University of Bradford, 2017)

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